

1 **++Running title:** Arabidopsis plants inhibit human breast cancer cells growth

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3 **Jasmonates induce Arabidopsis bioactivities selectively inhibiting the growth of breast**  
4 **cancer cells through CDC6 and mTOR**

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37 **SUMMARY**

38 Phytochemicals are often used *in vitro* and *in vivo* in cancer research. The plant hormones  
39 jasmonates (JAs) control the synthesis of specialized metabolites through complex regulatory  
40 networks. JAs possess selective cytotoxicity in mixed populations of cancer and normal cells.  
41 Here, direct incubation of leaf explants from the non-medicinal plant *Arabidopsis thaliana* with  
42 human breast cancer cells, selectively suppresses cancer cell growth. High -throughput LC-  
43 MS identified *Arabidopsis* metabolites. Proteins and transcript levels of cell cycle regulators  
44 were examined in breast cancer cells.

45 A synergistic effect by methyljasmonate (MeJA) and by compounds upregulated in the  
46 metabolome of MeJA treated *Arabidopsis* leaves, on the breast cancer cell cycle, is associated  
47 with CDC6, CDK2, CYCD1 and CYCD3, indicating that key cell cycle components mediates  
48 cell viability reduction. Bioactives such as indoles and quinolines and OPDA, in synergy, could  
49 act as anticancer compounds.

50 Our work suggests a universal role for MeJA-treatment of *Arabidopsis* in altering the DNA  
51 replication regulator CDC6, supporting conservation, across kingdoms, of cell cycle regulation,  
52 through the crosstalk between the target of rapamycin, mTOR, and JAs.

53 This study has important implications to identify metabolites with anti-cancer bioactivities in  
54 plants with no known medicinal pedigree and it will have applications in developing disease  
55 treatments.

56

57 *Key words:* *Arabidopsis thaliana*; bioassay; cancer therapy; cell cycle, jasmonate, natural  
58 compounds

59

60

## 61 INTRODUCTION

62 Plants produce many small molecules used as pharmaceuticals, insecticides, flavours, and  
63 fragrances with commercial applications which derive from their common use in defence  
64 against biotic challenges (Pérez-Salamó et al., 2019).

65 The ubiquitous plant stress hormone jasmonic acid (JA) and its oxylipin derivatives, like  
66 methyljasmonate (MeJA) and jasmonate isoleucin (JA-Ile), namely jasmonates (JAs) here, are  
67 potent regulators of plant defence, response to abiotic stress and developmental processes  
68 (Guo et al., 2018; Howe et al., 2018; Kazan, 2015; Pérez-Salamó et al., 2019; Riemann et al.,  
69 2015; Züst and Agrawal, 2017). Environmental pressures induce endogenous JAs  
70 biosynthesis. JA signalling triggers complex responses in plant cells, including massive  
71 transcriptional and metabolic reprogramming, and defence proteins and protective specialized  
72 metabolites biosynthesis (Balbi and Devoto, 2008; Bömer et al., 2018; Noir et al., 2013;  
73 Pauwels et al., 2009; Pérez-Salamó et al., 2019; Wasternack and Hause, 2013).

74 JAs control specialized metabolites synthesis through complex gene regulatory networks to  
75 limit it to when necessary. JAs induce most classes of specialized metabolites, including  
76 alkaloids, terpenoids, glucosinolates, and some phenylpropanoids (Balbi and Devoto, 2008;  
77 Pérez-Salamó et al., 2019; Zhou and Memelink, 2016). The precursor of JA, cis-(+)-12-  
78 oxophytodienoic acid (OPDA), also induces JA-independent specialized metabolites  
79 (Wasternack and Hause, 2013). Primary and secondary sulphur-related pathways leading to  
80 the synthesis of glucosinolates, have been shown to be MeJA responsive in *Arabidopsis* (Jost  
81 et al., 2005). Moreover the production of several agricultural and medicinal compounds,  
82 including glucosinolates, occurs through tryptophan metabolism (Smolen et al., 2002). The  
83 cabbage (*Brassica*) family, which includes *Arabidopsis thaliana*, is a rich source of  
84 glucosinolates and most biological activities for these in both plants and animals, reside with  
85 their cognate hydrolytic products. The isothiocyanates, such as sulforaphane, are outstanding  
86 examples (Dinkova-Kostova and Kostov, 2012).

87 Humans have long used plant-derived specialized metabolites as phytopharmaceuticals. Many  
88 phytochemicals have been identified as bioactive, including the prominent JA-induced  
89 anticancer drug, taxol (Baldi and Dixit, 2008). Fingrut and Flescher (2002) showed that JAs  
90 are potential anti-cancer agents (Fingrut and Flescher, 2002). JAs showed selective  
91 cytotoxicity in mixed populations of cancer and normal cells from chronic lymphocytic  
92 leukaemia patients (Fingrut and Flescher, 2002; Flescher, 2007). MeJA induced apoptotic  
93 death in cancer cells and the survival rates of mice bearing lymphoma were higher following  
94 MeJA treatment (Fingrut and Flescher, 2002). JAs and synthetic analogues exhibit anti-cancer  
95 activity in human breast, cervix, colon, colorectal, gastric, hepatoma, lung, lymphoma,

96 melanoma, myeloid leukaemia, neuroblastoma, prostate and sarcoma cancer cells (Balbi and  
97 Devoto, 2008; Cesari et al., 2014; Pérez-Salamó et al., 2019).

98 Three different mechanisms of action were proposed; bio-energetic, re-differentiation and  
99 reactive oxygen species (ROS)-mediated mechanisms to explain the activity of JAs against  
100 cancer cells (Flescher, 2007). MeJA has powerful anti-cancer activities both *in vitro* and *in vivo*,  
101 (Cohen and Flescher, 2009; Elia and Flescher, 2013; Fingrut and Flescher, 2002; Fingrut et  
102 al., 2005; Flescher, 2007; Li et al., 2017; Peng and Zhang, 2017; Rotem et al., 2003). JAs  
103 induce both apoptotic and non-apoptotic cancer cell death, independent of their p53 status,  
104 acting directly and selectively on mitochondria in cancer cells (Fingrut et al., 2005; Rotem et  
105 al., 2005). MeJA causes bio-energetic dysregulation and cell cycle arrest in different cancer  
106 cell types (Li et al., 2017; Rotem et al., 2005). MeJA treatment causes G0/G1 and S-phase  
107 arrest and induces apoptosis by increasing expression of tumour necrosis factor receptor 1  
108 (TNFR1), activation of mitogen-activated protein kinase (MAPK) and caspase-8, and  
109 decreasing the mitochondrial membrane potential in MCF-7 breast cancer cells (Yeruva et al.,  
110 2008). In non-small cell lung cancer cells, MeJA induces apoptosis (Zhang et al., 2016) and  
111 exerts its anticancer activity through downregulation of enhancer of zeste 2 polycomb  
112 repressive complex 2 subunit (EZH2), a histone methyltransferase, and the catalytic subunit  
113 of polycomb repressive complex 2 (PRC2) (Fu et al., 2014). Taken together these findings  
114 suggest that some of MeJA's anti-cancer activities are mediated by compounds upregulated  
115 by MeJA, although so far, the mechanisms of action of JAs and their induced metabolites on  
116 cancer cells, have never been compared.

117 Here, direct incubation of leaf explants of the non-medicinal plant *Arabidopsis thaliana* with  
118 human breast cancer cells is established in a bioassay comparing the efficacy of JA-regulated,  
119 specialised metabolites and MeJA on breast cancer cell lines. Metabolite extracts derived  
120 directly from the bioassay, including media and cancer cell controls, as well as wild-type and  
121 mutant plants, proved to be effective in the search for plant-derived, JA-induced specialized  
122 metabolites with anti-cancer activities. This system demonstrated consistently, the biological  
123 activity of plant material subjected to JA treatment on the growth inhibition of breast cancer  
124 cells. *Arabidopsis* mutants allowed dissection of the plant mechanisms controlling these  
125 bioactivities. The bioactivity of MeJA-treated, *Arabidopsis* leaf samples on the growth of breast  
126 cancer cells was COI1-dependent and mediated by JA induced plant-derived specialized  
127 metabolites such as indoles, quinolines and OPDA. The inhibitory effect was far superior to  
128 that of MeJA alone. Clustering and *in silico* identification of plant-derived MeJA-induced and  
129 COI1-dependent metabolic features showed that the effects on breast cancers cells are  
130 unlikely to be ascribed to individual features and that cancer cells metabolism affects

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131 bioactivity. We showed that the post translational down-regulation of CDC6, CDK2, CYCD1  
132 and CYCD3 is part of the mechanism to reduce breast cancer cell viability. Our analysis  
133 supports conservation, across kingdoms, of the regulation of the cell cycle through crosstalk  
134 between the target of rapamycin, mTOR, and JAs.

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135 **MATERIALS AND METHODS**

136

137 **Plant leaf disk bioassay using human breast cancer cells**

138 The antiproliferative effect of Arabidopsis plants aged 11 days after sowing (DAS) +/- 50 µM  
139 MeJA for 24h was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium  
140 bromide (MTT) cell viability assay.

141 MDA-MB-361, T-47D and MCF-10A were seeded in 96-well plates with 15,000 cells per well  
142 in a final volume of 100 µl medium and were left to set overnight. After 24 h, 3 x 1 mm  
143 (diameter) leaf disks excised from the first pair of true leaves were added aseptically to each  
144 well using a 1 mm Sample corer (InterFocus) and co-incubated with the cells for 72 h. Relative  
145 quantification of the cell proliferation of the human breast cancer cell lines T-47D and MDA-  
146 MB-361 and the non-tumourigenic cell line MCF-10A was assessed by MTT assay. For all  
147 treatments, the leaf disks and culture media were removed after the 72 hour incubation period  
148 and the MTT reagent was added to the wells in fresh medium.

149 Cytotoxic effects of MeJA on the cells were evaluated using a trypan blue inclusion assay

150

151 **Western blotting**

152 Total protein was extracted from the cell pellets using the NucleoSpin RNA/Protein Kit  
153 (Macherey-Nagel), and concentration determined using the Protein Quantification Assay Kit  
154 (Macherey-Nagel) and a microplate photometer (Multiskan EX Thermo Scientific). Equal  
155 amounts of protein (10ug) were resolved by SDS-PAGE. Protein bands were visualised using  
156 chemiluminescence detection systems Supersignal West Pico (Thermo Scientific) or Substrat  
157 HRP Immobilon Western (Merck Millipore) following manufacturer's instructions.

158

159 **Cell cycle analysis**

160 Ploidy levels were determined by flow cytometry using a Ploidy Analyser PAS (Partec GmbH),  
161 with UV excitation at 366 nm from a mercury arc lamp. Nuclei were released using Cystain  
162 extraction buffer (Partec), filtered through a Cell trics filter (Partec), and stained with Cystain  
163 fluorescent buffer (Partec). At least fifteen thousand nuclei were used for each ploidy  
164 measurement and the percentages of cells in the different phases of the cell cycle was  
165 calculated.

166

167 **Quantitative RT-PCR (qRT-PCR)**

168 Analysis of total RNA yield was performed on a nanodrop spectrophotometer (Labtech, UK).  
169 cDNA preparation was performed using the QuantiTect Reverse Transcription kit (Qiagen).

170 Real-time amplification was performed using SYBR Green JumpStart (Sigma-Aldrich)  
171 according to the manufacturer's instructions. Transcript analysis was performed from RNA  
172 samples derived from at least five independent experiments. The primer sequences are listed  
173 in Supplemental Table I.

174

#### 175 **Metabolite profiling by liquid chromatography-mass spectrometry (LC-MS/MS)**

176 Metabolite profiling was performed using a QToF (Quadrupole Time of Flight) 6520 mass  
177 spectrometer (Agilent Technologies, Palo Alto, CA, USA) coupled to a 1200 series Rapid  
178 Resolution HPLC system.

179

#### 180 **Data extraction and processing**

181 The raw data files (Agilent \*.d) of leaf disc-containing samples were processed with Mass  
182 Profiler (Version B.08.00, Agilent, Palo Alto, CA, USA) to extract features of interest (FOIs)  
183 using the built-in molecular feature extraction algorithm. Differentially expressed features were  
184 identified by 3-way ANOVA ( $p < 0.05$ ) using the Benjamini-Hochberg multiple comparison  
185 correction. This list was used for cluster and heat map generation. To lead the discovery of  
186 JA-regulated specialized metabolites with potential in inhibiting human breast cancer cell  
187 growth, we run linear regression models on the normalized (zero mean and unit variance) log<sub>2</sub>  
188 transformed abundances of each metabolite (total of 1757) with subsequent tests of a priori  
189 defined treatment contrasts (Hothorn *et al.*, 2008). These tests served as filtering conditions  
190 and metabolites that met them, were aggregated into corresponding sets (Table 1). All tests  
191 were performed with at a significance level of  $P = 0.05$ .

192 For further analysis, including medium only and medium plus T-47D cells, the raw data files  
193 were aligned and subjected to recursive molecular feature extraction using ProFinder (Version  
194 B.10.00, Agilent, Palo Alto, CA, USA). The resulting set of compounds were exported to  
195 MassProfiler Professional (Agilent) and analysed to identify plant specific and MeJA-induced  
196 features.

197 Where available MS/MS spectra of FOIs were extracted from raw data files using MassHunter  
198 Qualitative Analysis software (version B07.00) and compared with MS/MS data from Metlin  
199 and MassBank to provide putative identifications. The identification of JA and OPDA was  
200 further confirmed by comparison of retention time and spectra with standards. The  
201 stereoisomers of these compounds were not resolved by the chromatographic method used.  
202 Predicted MS/MS spectra were generated with the MetFrag tool ([https://msbi.ipb-](https://msbi.ipb-halle.de/MetFrag)  
203 [halle.de/MetFrag](https://msbi.ipb-halle.de/MetFrag)) (Ruttkies *et al.*, 2016).



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204 The data integral to the paper (fully documented LCMS/MS analysis) is available through  
205 <https://royalholloway.figshare.com/>. The DOI is 10.17637/rh.13079153.

206

207 **For further details and information on plant materials, human breast cancer cell lines,**  
208 **treatment, antibodies and analysis see** Supporting Information Methods S1.

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210 **RESULTS**

211  
212 **Methyljasmonate inhibits the growth of breast cancer but not that of non-tumorigenic**  
213 **cells**

214 The activity of MeJA was compared in human breast cancer cell lines T-47D, MDA-MB-361,  
215 with the non-tumourigenic mammary cell line MCF10A (Figure 1A). Both T-47D and MDA-MB-  
216 361 cell lines are ductal and oestrogen receptor (ER) positive with MDA-MB-361 expressing  
217 HER2 (Keydar et al., 1979). Previously, low or no JA-induced cytotoxicity in healthy cells were  
218 reported (Fingrut and Flescher, 2002; Reischer *et al.*, 2007; Rotem *et al.*, 2005; Rotem *et al.*,  
219 2003; Tong *et al.*, 2008).

220 Relative quantification of cell numbers, in response to increasing MeJA concentrations  
221 (according to Cesari et al 2014) was assessed, and dose-response curves obtained.  
222 Concentrations of 200  $\mu$ M and 2 mM MeJA significantly inhibited T-47D cells growth. MDA-  
223 MB-361 cell growth was also significantly suppressed at 2 mM MeJA, but less than T-47D  
224 (Relative cell number (RCN) 78% and 47%, respectively). The leaf disks and culture media  
225 were removed after the 72 hour incubation period and the 3-(4,5-dimethylthiazol-2-yl)-2,5-  
226 diphenyltetrazolium bromide (MTT) reagent was added to the wells in fresh medium to avoid  
227 interference of any compound released by the leaf explants or MeJA that could have led to the  
228 reduction of MTT to formazan (through differential regulation of enzyme activity). The effects  
229 seen are therefore a direct result of changes in cell number rather than in enzyme activity. The  
230 dose response curves were used to calculate the half maximal inhibition concentration (IC<sub>50</sub>)  
231 values and these were determined as 1.87mM, 4.44mM and 5.14mM respectively for T-47D,  
232 MDA-MB-361 and MCF-10A.

233 The effect of MeJA on cell cycle progression was determined by flow cytometry: treatment  
234 resulted in T-47D cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> (Supplemental Figure 1 B-E). Non-tumourigenic  
235 MCF-10A cells were not significantly affected by MeJA or treated Arabidopsis (Figure 1A and  
236 Supplemental Figure 1 A-E). Cell death was also only mildly affected at concentrations from  
237 200 $\mu$ M or higher (Supplemental Figure 1 A), demonstrating that MeJA had more profound  
238 effects on survival of the tumour cell lines.

239  
240  
241 **A bioassay of activity of leaf disks on different breast cancer and non-tumorigenic cell**  
242 **types**

243 We assayed the effects of MeJA treatment of Arabidopsis leaves before explants were taken,  
244 on the growth of breast cancer cells T47-D and MDA-MB361 and on non-tumourigenic MCF10-

245 A. *Arabidopsis thaliana* mutants impaired in JA biosynthesis or signal perception were tested  
246 (Figure 1 B, C and D): the *coi1-16B* mutant for the JA receptor COI1, displaying a JA-  
247 insensitive phenotype (Ellis and Turner, 2002; Noir et al., 2013); the transgenic COV99, over-  
248 expressing line the JA receptor COI1 (Devoto et al., 2002); the CONSTITUTIVE EXPRESSION  
249 OF VSP1 (*cev*), with higher levels of JAs (Ellis and Turner, 2001); the allene oxide synthase  
250 (*aos*) knock-out mutant, defective in the JA biosynthetic gene CYP74A (AOS) (Park et al.,  
251 2002), unable to produce JAs but capable of JA responses. All JA mutants, except for *coi1-*  
252 16B showed a clear phenotypic response to the 24h MeJA treatment including some visible  
253 effects on the growth (Figure 1B), previously associated with JA treatment (Noir et al., 2013;  
254 Shan et al., 2009). The effectiveness of the JA treatment was also confirmed through the  
255 expression of JA-responsive genes such as *Vegetative storage protein (VSP)* 1 and 2 and  
256 AOS (data not shown). T-47D cells, treated with MeJA or wild-type (WT) *Arabidopsis* were  
257 observed under brightfield (Supplemental Figure 2). Incubation with untreated leaf disks visibly  
258 decreased cell density, inducing rounder morphology and increasing floating debris. These  
259 changes were consistent with increased cell death (Supplemental Figure 1) A).  
260 The use of a bioassay, originally devised to analyse chemopreventive glucosinolates in murine  
261 hepatoma cells (Wang et al., 2002) was extended to human cancer cell lines and further  
262 modified to test cell viability with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium  
263 bromide). Direct analysis of the effects of single leaf disks from the *Arabidopsis Col gl1* (wild-  
264 type, WT) or mutants was performed (Figure 1C). Suppression of tumour cell growth was  
265 consistently the strongest when cells were co-incubated with MeJA-treated plant samples  
266 compared to the untreated control plant disks, except for *coi1-16B* (Figure 1D). *Arabidopsis*  
267 explants treated with 50  $\mu$ M MeJA showed an inhibitory effect comparable to the treatment  
268 with the highest (mM) concentrations of MeJA (Figure 1A and D). Both T-47D and MDA-MB-  
269 361 cancer cell lines showed comparable responses (Figure 1D). RCN Inhibition values for  
270 MeJA-treated plant leaf samples compared to the untreated controls are shown in  
271 Supplemental Table II. The growth of T-47D was reduced to 68% RCN when exposed to  
272 untreated WT disks but significantly more reduced (36% RCN  $P < 0.001$ ) when exposed to  
273 MeJA-treated disks (Figure 1D). This compares to 68% and 33% RCN respectively for MDA-  
274 MB-361 cells. Mutant *aos* reduced the cell growth of T-47D to 71% and 47% RCN for untreated  
275 and MeJA-treated plant leaf disks, respectively. This compares to 92% and 50% RCN for MDA-  
276 MB-361 cells (Supplemental Table II). The *aos* RCN inhibition values of 33% for T-47D and  
277 46% for MDA-MB-361 cells were slightly but not significantly lower when compared to WT  
278 (Supplemental Table II). A wild-type-like effect was exerted by COV99 samples with 69% to  
279 41% RCN for untreated and treated disks when testing T-47D cells and 80% and 40%

280 respectively for MDA-MB-361. The inhibition of both breast cancer cell lines by *cev1* untreated  
281 and treated leaf disks was higher than that caused by WT. RCN values reached 46% to 20%  
282 and 59% to 17% for untreated and treated samples when testing T-47D and MDA-MB-361  
283 cells, respectively. Consequently, inhibition values of *cev1* samples were also found to be  
284 higher compared to WT samples (Supplemental Table II), consistent with the presence of  
285 elevated levels of endogenous JAs. For all plant samples tested, co-incubation with excised  
286 leaf disks resulted in significantly lower RCN values when testing the growth of T-47D and  
287 MDA-MB-361 cancer cells. Remarkably, the growth of non-tumourigenic MCF-10A cells was  
288 not significantly affected, except for MeJA-treated *cev1*, which has constitutive JA responses  
289 (Ellis and Turner, 2001).

290 The differential effect between MeJA-treated and untreated plant samples was significant for  
291 all *Arabidopsis* mutant lines tested except for *coi1-16B* (Figure 1D). MeJA-treated *coi1-16B*  
292 samples did not reduce the cell growth of either cancer cell line further when compared to the  
293 untreated *coi1-16B* controls showing that the observed differential effect between MeJA-  
294 treated and untreated leaf samples on the breast cancer cell growth was *COI1*-dependent, as  
295 reflected in significantly lower inhibition values compared to the Col *gl1* WT (Supplemental  
296 Table II). We also tested the effect of *Arabidopsis* mutants impaired in glucosinolates or  
297 tryptophan metabolism on T47-D and MCF10A cells (Supplemental Figure 3 and  
298 Supplemental Table II). These mutants showed no obvious or significant differential effects on  
299 the growth of the T-47D or the non-tumourigenic MCF-10 lines compared to their Col-0 WT.  
300 In summary, the results obtained for *coi1-16B* were consistent with the role of *COI1* in the JA  
301 signalling pathway. However, higher expression of the JA receptor in *COV99* (Bömer et al.,  
302 2018) did not cause any additional effects. The mutant *aos*, lacking the positive feedback loop  
303 amplification of the JA signal (Park et al., 2002), showed a less pronounced, though not  
304 significantly different, effect compared to the WT. In accordance with the JA-dependency of  
305 the observed effects on breast cancer cells, *cev1*, which has constitutive JA responses (Ellis  
306 and Turner, 2001), displayed the strongest inhibitory potential.

### 307 308 **Metabolite profiling of human breast cancer T-47D cell culture media after incubation** 309 **with *Arabidopsis***

310 High throughput metabolic profiling to investigate the effects of JAs on *Arabidopsis* plants in  
311 isolation has been performed previously, albeit in different mutants and experimental  
312 conditions, on leaf intracellular extracts, and multivariate statistical analyses performed to  
313 obtain compound libraries (Cao et al 2016). Active compounds from plants need identification  
314 and mechanisms of action characterised to assess the full potential of the bioactives for clinical

315 trials and applications, efficiency and any adverse side effects. The inhibitory effects of MeJA-  
316 treated Arabidopsis explants on human breast cancer cells growth, encouraged a search for  
317 specialized metabolites using untargeted LC-MS/MS metabolic profiling. Candidate bioactive  
318 MeJA-inducible compounds were predicted to be more abundant in medium of MeJA-treated  
319 WT treatments than with *coi1-16B*. We focussed our analysis on compounds present in the  
320 cell media that could be recognised by surface receptors. Molecular feature extraction using  
321 MassProfiler identified co-eluting isotopes and adducts comprising 1757 putative features of  
322 interest (FOIs) in positive and negative ion modes (Supplemental Table III). Principal  
323 component analysis (PCA) based on the relative abundance of these FOIs showed clear  
324 discrimination between (i) the T-47D and no cell backgrounds; (ii) the Arabidopsis wild-type  
325 and *coi1-16B* plant leaf disks; (iii) the MeJA treatment in the Col *gl1* samples (Figure 2A).  
326 Hierarchical clustering of all FOIs based on their normalized metabolite abundances, resulted  
327 in well-defined clusters (Figure 2B). Distinct metabolite profiles could be detected in the  
328 incubation medium from different treatments. The individual features were further filtered using  
329 predefined conditions (Table I), identifying 146 FOIs (Figure 2C) which responded to MeJA in  
330 Col *gl1* (A1) more than in *coi1-16B* (B1) and were similarly abundant in untreated Col *gl1*  
331 compared to untreated *coi1-16B* (C1). These features fell into clusters 1 and 2 (Figure 2B)  
332 representing compounds affecting the viability of the T-47D cells. Clusters 1 and 2 identified  
333 69 FOIs present or absent in the no T-47D cell control samples, respectively. Cluster 2 FOIs  
334 were *COI1*-dependent, MeJA-induced and produced in the Col *gl1* WT background  
335 independently of the presence or absence of T-47D cancer cells and therefore, plant-derived.  
336 In contrast, Cluster 1 FOIs were *COI1*-dependent, MeJA-induced and occurred in the Col *gl1*  
337 WT background, but only in the presence of T-47D cancer cells. Further filtering (Table I)  
338 identified FOIs MeJA-induced in *coi1-16B* (10 FOIs), and those more/less abundant in the T-  
339 47D cell background. These are likely to represent end- or by-products of T-47D metabolism  
340 (117 FOIs), compared to cancer cell media compounds, metabolised by the T-47D cells (98  
341 FOIs), respectively.

342 Further analysis identified plant derived Col *gl1*-specific MeJA-induced features. All samples,  
343 including medium-only and medium plus T-47D cells were re-aligned and molecular feature  
344 extraction using MassHunter Profinder followed by statistical analysis with MassProfiler  
345 Professional, performed. This analysis extracted 161 and 105 putative features in positive and  
346 negative ion mode respectively which were present exclusively in samples containing leaf  
347 discs (Supplemental Table IV). Of these plant-specific features, 21 (15 and 6 in positive and  
348 negative ion mode respectively) were induced by MeJA in Col *gl1* but not in *coi1-16B*  
349 (Supplemental Table IV).

350

### 351 **Discovery of bioactive metabolites**

352 Accurate mass and isotope composition for the 161 plant-specific putative features were used  
353 to calculate molecular formulas, search databases and (where present), MS/MS spectra were  
354 extracted. Several identifiable compounds were detected based on accurate mass and MS/MS  
355 spectral matches (Supplemental Table V). The abundance of these compounds was compared  
356 in samples containing Arabidopsis wild-type (Col *gl1*) or mutant *coi-16B* in the presence or  
357 absence of T-47D cells and/or MeJA against media only (m) As expected, increased JA was  
358 detected in MeJA-treated samples in a COI1-independent manner (Figure 3). JA presence  
359 was unaffected by the presence of T-47D cells. Of the major specialized Arabidopsis  
360 metabolites, 4-methylsulfinylbutyl isothiocyanate (sulforaphane) a breakdown product of the  
361 glucosinolate glucoraphanin (Kissen et al., 2009) and the flavonol glycoside kaempferol  
362 hexoside deoxyhexoside, belonging to one of the major Arabidopsis flavonoids (Veit and Pauli,  
363 1999), were identified. Kaempferol glycoside abundance was not affected by MeJA or by the  
364 presence of T-47D cells and its presence was COI1-independent. Sulforaphane levels were  
365 mildly induced by MeJA in Col *gl1* albeit at lower levels in *coi-16B* but more strongly in the  
366 presence of T-47D. 12-oxo-phytodienoic acid (OPDA), a biosynthetic precursor of JA  
367 (Zimmerman and Feng, 1978) was detected. OPDA accumulated in MeJA-treated samples  
368 and at a lower level in *coi-16B*, but the levels were dramatically reduced in presence of T-47D  
369 cells. Of the compounds showing specific induction by MeJA only in Col *gl1*, hence COI1-  
370 dependent, one (3 Pos, retention time 10.78 minutes; Figure 3, Supplemental Tables IV and  
371 V) allowed preliminary identification. Its abundance was decreased by the presence of T-47D  
372 cells. Its predicted formula C<sub>10</sub>H<sub>9</sub>N<sub>0</sub>/159.0864 (measured 159.0679, 11 ppm deviation)  
373 corresponded to several candidate compounds, although it was outside the observed mass  
374 accuracy for the other identified compounds and the umbelliferone internal standard (5 ppm).  
375 In the MS/MS spectrum, 117.06 m/z is characteristic of indoles (e.g. indole acetaldehyde) and  
376 quinolines (e.g. 2-methyl-4-hydroxyquinoline and others).  
377 Further analysis is required to precisely identify the active ingredients in the complex plant  
378 mixture, the compounds detected in Arabidopsis through this study were potential bioactive  
379 compounds.

380

381

### 382 **Incubation with MeJA-treated Arabidopsis altered transcripts and protein levels of cell** 383 **cycle regulators in breast cancer cells**

384 Given that MeJA (Figure 1A) or MeJA-treated *Col gl1* (Figure 1C) reduced T-47D cell numbers  
385 and that MeJA influences cell cycle progression (Supplemental figure 1 B-E), the effects on  
386 cell cycle markers were investigated.

387 We selected the following G1/S specific regulators (Caldon and Musgrove, 2010; Cohen and  
388 Flescher, 2009) and tested their relative gene expression and protein abundance: Cyclin-  
389 dependent kinase 2 (CDK2, P24941), Cyclin D1 (CCND1, P24385), Cyclin D3 (CCND3,  
390 P30281), Cyclin E1 (CCNE1, P24864), cell division cycle 6 (CDC6, Q99741), proliferating cell  
391 nuclear antigen (PCNA, P12004), Cyclin-dependent kinase inhibitor 1A (p21, Cip1, CDKN1A,  
392 P38936) and Cyclin-dependent kinase inhibitor 1B (p27, Kip1, CDKN1B, P46527). Cell cycle  
393 progression through G1/S is mediated by Cyclin D/CDK4 or CDK6 and Cyclin E/CDK2 protein  
394 complexes and, once in S phase, CDC6 and PCNA are essential for DNA replication (Matson  
395 and Cook, 2017). On the other hand, the Cyclin-dependent kinase inhibitors p21 and p27  
396 prevent Cyclin/CDK complex formation and participate in DNA damage repair (Abukhdeir and  
397 Park, 2009).

398 The protein levels of CDC6 and CDK2 in cells in response to MeJA remained unchanged  
399 (Figure 4A), but the gene expression levels decreased (Supplemental Figure 4). Cyclin D1,  
400 Cyclin D3, p27 and PCNA protein levels mirrored the changes in transcript levels and were  
401 unaffected by MeJA compared to levels in lysates from the mock, ethanol-treated cells. Cyclin  
402 E1 protein levels were reduced and both p21 transcript and protein levels increased in cells  
403 treated with MeJA, compared to controls (Figure 4A and Supplemental Figure 4).

404 When cells were co-incubated with leaf disks from MeJA-treated plants, protein and transcript  
405 levels of CDC6, Cyclin D1 and CDK2 were reduced compared to cells incubated with untreated  
406 leaf disks. Cyclin D3 protein levels were also reduced, although no differences were observed  
407 in transcripts. Co-incubation with MeJA-treated *Col gl1* leaf disks also increased *Cyclin E1*,  
408 *p21* and *p27* transcripts, although, the effects on protein levels differed; Cyclin E was  
409 increased, p21 was decreased and p27 levels were unaffected. PCNA levels were unaffected  
410 at either transcript or protein levels (Figure 4A and Supplemental Figure 4).

#### 411 412 **The effect of MeJA-treated Arabidopsis on the mechanistic Target of Rapamycin** 413 **(mTOR) signalling pathway**

414 The mechanistic Target of Rapamycin (mTOR) is a known therapeutic target in breast cancer  
415 (Hare and Harvey, 2017). Recent evidence suggests crosstalk between TOR and JA signalling  
416 in Arabidopsis (Pérez-Salamó et al., 2019; Song et al., 2017). For this reason, the effects of  
417 both MeJA and MeJA-treated *Col gl1* on mTOR in T-47D breast cancer cells were examined.  
418 (Figure 4B). Notably, the treatment of T-47D cells with MeJA caused the opposite effects to

419 those observed with the leaf disks. An increase in the protein levels of mTOR, p-TOR  
420 (Ser2481), p-TOR (Ser2448), RICTOR, RAPTOR and G $\beta$ L was observed in MeJA-treated  
421 cells compared to untreated controls. In contrast, incubation with MeJA-treated Col *gl1* did not  
422 affect the protein levels of mTOR and RAPTOR, but decreased the protein abundance of  
423 phosphorylated mTORC p-TOR (Ser2448), p-TOR (Ser2481), RICTOR and G $\beta$ L in T-47D cells  
424 in comparison to cells incubated with the untreated leaf disks (Figure 4B).

425 Overall, these data showed that both MeJA and MeJA-treated WT Arabidopsis leaves affect  
426 mTOR protein levels. When combined with the differences in cell cycle protein data (Figure  
427 4A) they indicate that the component(s) involved in mediating the bioactivities on breast cancer  
428 cells are likely to be compound(s) or downstream metabolite(s) from MeJA-treated leaf  
429 explants, distinct from those induced in T-47D cells by direct treatment with MeJA (or indeed  
430 MeJA itself).

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432 **DISCUSSION**

433 **A bioassay to assess the effect of MeJA on the growth of human breast cancer cells**

434 A systematic relationship between the production of plant specialized metabolites (Balbi and  
435 Devoto, 2008; Pérez-Salamó et al., 2019; Zhou and Memelink, 2016) and the regulation of  
436 growth and development and response to stress has not yet been established. Moreover,  
437 Arabidopsis, despite having been used as a model plant for over 30 years, has never been  
438 considered as a potential source of phytotherapeutics.

439 JAs elicit *de novo* transcription and translation and, ultimately, the biosynthesis of specialized  
440 metabolites in plants (Memelink et al., 2001). The anti-cancer activity of JAs has been  
441 demonstrated both *in vitro* and *in vivo* (Balbi and Devoto, 2008; Cesari et al., 2014; Fingrut  
442 and Flescher, 2002; Flescher, 2005; Pérez-Salamó et al., 2019). However, it is not known  
443 whether this is a direct effect of JAs. Induction of specialized metabolites could underlie the  
444 increased growth inhibition of human breast cancer cells when co-incubated with MeJA-treated  
445 plant samples. The effectiveness of compounds from plants could also be affected by  
446 interaction between them and the target cells. To our knowledge, there has been no systematic  
447 study comparing the effect of JAs on breast cancer and non-tumourigenic breast cells.

448 We show that the direct cytotoxic effect of MeJA is selective for human breast cancer cells  
449 (Figure 1A; Supplemental Figure 1). Previous studies have reported low or no cytotoxicity of  
450 JAs to healthy cells compared to cancer cells (Fingrut and Flescher, 2002; Reischer et al.,  
451 2007; Rotem et al., 2003). Cytotoxicity assays (Yeruva et al., 2008) showed a significant  
452 decrease in the cell viability of the human breast cancer cell lines MDA-MB-435 and MCF-7 at  
453 concentrations of 1.5 mM MeJA and higher.

454 In our study, Arabidopsis explants treated with 50  $\mu$ M MeJA had an effect on breast cancer  
455 cells (Figure 1D) comparable to treatment with mM concentrations of MeJA. Such enhanced  
456 efficacy could be indicative of synergic effects of JAs with other compounds. The reduced  
457 sensitivity of MDA-MB-361 compared to T-47D cells, could be attributable to their HER2  
458 positivity, linked to recalcitrance to chemotherapy (Sauter et al., 2009). This difference  
459 emphasizes the usefulness of our bioassay to detect differences between treatments and cell  
460 types and to detect the interactions between phytotherapeutics and cancer cells.

461  
462 **COI1-dependent JA signalling mediates the effect of exogenous MeJA treatment of**  
463 **Arabidopsis explants on reducing human breast cancer cells growth**

464 Suppression of cancer cell growth was consistently stronger when cells were co-incubated  
465 with MeJA-treated plant samples compared to untreated controls (Figure 1D). The differential  
466 effect between MeJA-treated and untreated plant samples was due to JA signalling. This

467 finding indicated that anti-cancer activity was not only a direct effect of MeJA itself, but the  
468 result of production of JA-regulated, COI1-dependent specialized metabolites (Devoto et al.,  
469 2005; Pauwels et al., 2008; Pérez-Salamó et al., 2019).

470 COI1 over-expression was previously found to affect positively the availability of metabolites  
471 such as  $\beta$ -alanine, threonic acid, putrescine, glucose and myo-inositol, thereby providing a  
472 connection between JA-inhibited growth and stress responses (Bömer et al., 2018). Here,  
473 COV99 plants over-expressing the COI1 receptor, exhibit wild-type responses in bioassays,  
474 indicating that any observed increases in inhibition of cancer cell growth by MeJA-treatment of  
475 Arabidopsis do not necessarily depend on the dose of the COI1 receptor.

476 The differential effects of MeJA treated and untreated leaf samples were consistently less  
477 pronounced in *aos* compared to the corresponding WT Col *gl1*, indicating that the positive  
478 feedback regulatory loop and endogenous JA levels may contribute to the anti-cancer potential  
479 of MeJA-treated Arabidopsis. In accordance with the JA-dependency of the effects on the  
480 breast cancer cells, explants of *cev1* displayed the strongest inhibitory potential on the growth  
481 of T-47D and MDA-MB-361 breast cancer cells. Higher endogenous levels of JAs and  
482 constitutive JA responses in the *cev1* mutant (Ellis and Turner, 2001) emphasize the role of  
483 JA-induced specialized metabolites in breast cancer cells growth suppression. The enhanced  
484 efficacy of the MeJA-treated samples in inhibiting the growth of breast cancer cells could also  
485 be indicative of a synergistic effect between JAs and the production of other plant-derived  
486 compounds with anti-cancer activity. Notably, cell growth of the non-tumourigenic MCF-10A  
487 cells was not affected significantly following exposure to leaf disks, except for exposure to  
488 MeJA-treated *cev1* explants. The inhibitory potential of untreated Arabidopsis leaf disks and  
489 leaf disks from MeJA-treated plants, is likely, therefore selective, for cancer cells  
490 demonstrating a similar selective cytotoxicity towards cancer cells as previously described for  
491 MeJA.

492

### 493 **Identification of Arabidopsis metabolites inhibiting breast cancer cell growth**

494 Untargeted LC-MS/MS identified COI1-dependent, and MeJA-induced compounds in the cell  
495 media, in presence and absence of T-47D cells (Figure 2 and Table I). This analysis indicated  
496 complex interactions between Arabidopsis metabolites and T-47D cells. 266 plant specific  
497 MeJA-induced features were detected (Supplemental Table IV) and some putatively identified,  
498 based on accurate mass and MS/MS spectral matches, lending confidence to our conclusion  
499 that others are unknowns awaiting discovery (Figure 3 and Supplemental Table V). The  
500 uniform abundance of the plant specific flavonol glycoside, kaempferol hexoside  
501 deoxyhexoside, across all samples (Veit and Pauli, 1999) validated compounds changes under

502 varying conditions. The identification of JA in MeJA-treated samples also validated our ability  
503 to identify possible breakdown products of MeJA and/or to detect endogenous JAs inhibiting  
504 breast cancer cells growth.

505 One explanation for the complex OPDA abundance pattern, induced in MeJA-treated samples  
506 and decreased in the presence of T-47D cells, is that MeJA inhibits endogenous JA synthesis  
507 in leaves, resulting in OPDA accumulation and secretion. Since there are four possible  
508 stereoisomers of OPDA in plants (Schaller *et al.*, 1998), it is possible that that MeJA induces  
509 accumulation of a specific one. Unfortunately, the chromatography column used for LC-MS is  
510 unable to resolve these stereoisomers. However, the T-47D cells might metabolize OPDA (or  
511 inhibit its secretion). Our data support OPDA induction of JA-independent specialized  
512 metabolite production (Taki *et al.*, 2005) and that direct treatment with OPDA has anticancer  
513 activity by targeting Cyclin D1 (Nedret. *et al.*, 2008). Significantly, we showed that OPDA is  
514 produced by the plants and that it may cause the reduction in the Cyclin D1 protein (Figure  
515 4A).

516 Although glucosinolates, including sulforaphane, act as anti-cancer compounds (Mokhtari *et al.*,  
517 2018), isothiocyanates derived from glucosinolates do not play a major role in growth  
518 suppression of the human breast cancer cells (Supplemental Figure 3, Supplemental Table II).  
519 The possible identification of a plant-specific MeJA-induced compound with an MS/MS  
520 spectrum characteristic of indoles and quinolines provides possible mechanistic insights into  
521 the effects of MeJA in plants. Importantly both classes of compounds have been previously  
522 identified as anticancer (Musiol, 2017) but their mechanisms of actions remained elusive.

523

524

#### 525 **D-type Cyclins CDC6, CDK2 are mechanistic targets of MeJA-induced Arabidopsis** 526 **bioactivities**

527 The effect on the growth of breast cancer cells caused by MeJA-treated plants, and by direct  
528 MeJA treatment (Figures 1, Supplemental Figure 3 and Supplemental Figure 2), prompted our  
529 investigation of cell cycle markers in T-47D cells, under both conditions, whereby strikingly  
530 differential effects were detected (Figure 4 and Supplemental Figure 4). Our data suggest that  
531 JAs delay the progression of cells from G0/G1 phase into S phase inducing apoptosis  
532 (Supplemental Figure 1C and D). Stalling the cells in G0/G1, may gain time to repair cellular  
533 damage. At high doses of MeJA, irreparable damage induced cell death. Similarly, efforts to  
534 increase G2-M arrest have been associated with enhanced apoptosis (Ehrlichová *et al.*, 2005).  
535 The action of D and E-type Cyclins, CDK2 and the CDK inhibitor proteins p21, p27 and p57  
536 characterise the G1 phase of the cell cycle and activation of target proteins for S phase

537 progression (Caldon and Musgrove, 2010). p27 and p21 inhibit Cyclin CDK complexes in  
538 G0/G1. It has been hypothesised that PCNA downregulation induces cell cycle arrest, in  
539 association with Cyclin D (D1 or D3), CDK2 and p21 (Cohen and Flescher, 2009). Lack of  
540 detectable changes in expression of either p27 and PCNA in our study suggests cell type-  
541 specific regulation of the cell cycle either by MeJA or by MeJA-treated leaf explants. The  
542 relative stability of p27 could also be a positive indicator of better patient outcome following  
543 MeJA treatment (Alkarain et al., 2004).

544 MeJA-treatment reduced Cyclin E1 and increased p21 protein levels (Figure 4 A), supporting  
545 our cell cycle analysis (Figure 4A). E-type Cyclins activity is limiting for cells passing from G1  
546 into S-phase. Cyclin E binds and activates CDK2 leading to S-phase specific gene expression  
547 (Möröy and Geisen, 2004). CDK2 also phosphorylates several components of the DNA pre-  
548 replication complex, including CDC6 (Chuang et al., 2009). The reduction of Cyclin E1 and  
549 increased p21 protein levels (Figure 4A, Figure 5), also supports the flow cytometry analysis.  
550 A different effect was observed for MeJA-treated plants on Cyclin E1 and p21, highlighting the  
551 mechanistic differences between the direct effects of MeJA treatment of the breast cancer cells  
552 and incubation with MeJA-treated plant explants.

553 Downregulation of CDK2 gene expression was observed following incubation with both MeJA  
554 and MeJA-treated leaf disks (Supplemental Figure 4), although only the latter caused reduction  
555 of CDK2 protein (Figure 4A, Figure 5). This suggests that incubation with MeJA-treated plant  
556 explants, but not with MeJA, activate additional signalling pathways leading to CDK2 protein  
557 degradation. The association of ubiquitin-dependent degradation of CDK2 with the arrest of  
558 tumour growth in acute myeloid leukemia (AML) cells (Ying et al., 2018) supports this  
559 hypothesis.

560 D-type Cyclins (Cyclin D1, D2 and D3) are essential for G1 phase and can limit G1/S transition  
561 (Herzinger and Reed, 1998). Our results confirmed that MeJA treatment of human breast  
562 cancer cell lines had no effect on Cyclin D1 expression at the RNA level as reported for  
563 neuroblastoma cells by Tong *et al.*, (2008). Strikingly, we also showed that MeJA-treated  
564 Arabidopsis explants substantially reduced the levels of cyclin D proteins in human breast  
565 cancer cell lines, in accordance with studies linking the down regulation of Cyclin D1 and D3  
566 levels to antitumor therapy in breast cancer patients (Ortiz et al., 2017; Wang et al., 2019),  
567 hereby providing mechanistic targets for MeJA-induced plant bioactivities inhibiting breast  
568 cancer cells growth, and further ground for these cell cycle regulators as targets of anticancer  
569 compounds.

570 The mammalian CDC6 is a trifunctional AAA+ ATPase (Duderstadt and Berger, 2008),  
571 controlling the G1/S transition, DNA replication and cell survival (Okayama, 2012). CDC6 also

572 controls CDK2 activity during G1/S transition and subsequent obstruction of apoptosome  
573 assembly inhibiting cell death during proliferating (Niimi et al., 2012). In our study, the levels  
574 of CDC6 protein are dramatically reduced by MeJA-treated plants, supporting previous findings  
575 where CDC6 was identified as target for radiosensitivity in nasopharyngeal carcinoma (Li et  
576 al., 2016). CDC6 down-regulation shares similarity with the effect of MeJA treatment on  
577 Arabidopsis we previously demonstrated (Noir et al., 2013). JAs-induced effects, common to  
578 both mammalian and plant cells following JA treatment, include the suppression of cell  
579 proliferation, ROS generation, cell death induction, HSP expression and MAPK induction (Balbi  
580 and Devoto, 2008; Cesari et al., 2014; Flescher, 2007; Pérez-Salamó et al., 2019). The list of  
581 common effects can be therefore extended to reduction in CDC6 activity, a key component in  
582 JAs-suppressed growth in both plant and cancer cells.

583  
584 **The inhibition of the mTORC2 complex is a target for MeJA induced plant bioactivities**

585 In breast cancer cells, treatment with Palbociclib, a CDK4/6 inhibitor, upregulates mTOR whilst  
586 promoting G0/G1 cell cycle arrest (Cretella et al., 2018). Cell cycle arrest in the presence of  
587 active mTOR promotes senescence and geroconversion, but the inhibition of mTOR with  
588 rapamycin partially suppresses the senescent phenotype (Leontieva and Blagosklonny, 2013).  
589 In our study, different effects were caused by MeJA treatment alone and by incubation with  
590 MeJA treated plant explants. It is surprising that, upon MeJA treatment, mTORC1/2 protein  
591 levels increased, while incubation with MeJA-treated leaf disks decreased the protein levels of  
592 the mTORC2 component Rictor (Figure 4B, Figure 5), highlighting mechanistic differences  
593 between the two treatments. The mTORC1 complex senses nutrient status to regulate protein  
594 and lipid biosynthesis, stimulating cell growth. The mTORC2 complex also responds to growth  
595 factors, as well as regulating the actin cytoskeleton, ion transport, and cell growth and survival  
596 (Jacinto et al., 2004). Our findings indicate that inhibition of the mTORC2 complex is the  
597 mechanism for MeJA induced plant bioactivities blocking breast cancer cells growth.

598 In Arabidopsis, in response to positive mitogenic signals, such as light, sugar availability, and  
599 hormones, TOR signalling pathway promotes cell growth that connects to the entry and cell  
600 division cycle via multiple signalling (Ahmad et al., 2019; Henriques et al., 2014). Yet there is  
601 no evidence of crosstalk between the effects of JAs and mTOR signalling in mammalian cells.  
602 However, in plants, mTOR is known to regulate phytohormone synthesis, as well as JAs  
603 signalling (Pérez-Salamó et al., 2019; Song et al., 2017), whereby crosstalk contributes to the  
604 trade-off between growth and defence, by modulating JA signalling and biosynthesis regulating  
605 growth conditions (Pérez-Salamó et al., 2019; Song et al., 2017).

606 Our study ascribes separate roles for MeJA and MeJA-derived compounds from Arabidopsis  
607 impacting mTOR in the breast cancer cell cycle. It is striking that conserved crosstalk between  
608 mTOR and JAs occurs in different kingdoms in regulating cell cycle. Further studies of these  
609 naturally occurring plant compounds will be important to improve our understanding of  
610 checkpoint modulation and potentially to develop novel clinical approaches to the treatment of  
611 human cancers.

612

## 613 **CONCLUSIONS**

614 Our findings ascribe unprecedented medicinal properties to what has been considered so far,  
615 a model plant, Arabidopsis. By studying the signalling in cancer cells we discovered universally  
616 conserved modes of action of JAs between plant and animal cells. Overall, in our study, a  
617 synergistic effect by MeJA and by compounds induced by it on the cell cycle associates with  
618 the decreased levels of CDC6, CDK2, CYCD1 and CYCD3 (Figure 5). Consequently, the  
619 down-regulation of these cell cycle regulators could mediate the mechanism behind the  
620 reduction in breast cancer cell viability. Strikingly for future applications in cancer therapy, the  
621 action of MeJA and compounds upregulated in Arabidopsis metabolome, target a central pivot  
622 of the highly complex mechanism controlling cell proliferation and survival. Whether the effect  
623 on cell cycle markers depends on mTOR, or on the activation of the latter by MeJA as a  
624 downstream cellular response, remains to be demonstrated.

625 The study provides a new platform for the discovery of plant derived, bioactive compounds  
626 within complex plant mixtures while also allowing the identification of synergistic effects  
627 between phytochemicals and target cells. Most traditional chemotherapeutic agents are non-  
628 specific but selective as they act by killing cells that divide rapidly, which is one of the main  
629 properties of most cancer cells. We validated the reproducibility of the system by undertaking  
630 assays with healthy epithelial cells; showing that MeJA-treated Arabidopsis explants are  
631 effective in selectively modulating the proliferation of tumourigenic compared to non-  
632 tumourigenic cells and discriminating between them.

633 The bioassay allowed production of high value chemicals in sufficient quantities to be detected  
634 by LCMS even from plants with no known medicinal pedigree, allowing mining of untapped  
635 resources without *a priori* assumptions. The system has the potential to be adapted to identify  
636 different classes of bioactive phytochemicals. Different plants can be tested allowing direct  
637 comparison of known medicinal plants with new ones with unrecognised effects; different cell  
638 types could be used to define the specificity of bioactive phytochemicals and assays could be  
639 calibrated with the combinatorial use of mutants or phytochemical inducers. The analysis of  
640 the metabolome within targets cells could also be performed to gain more insights on the

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641 absorption mechanisms. This study has important implications to identify metabolites with anti-  
642 cancer bioactivities and it will have applications in developing treatments for other diseases.  
643 Combined with recent progress in metabolic engineering and biotechnology, our approach will  
644 also facilitate production and analysis of bioactivities of valuable metabolites from plants on  
645 industrial scales.  
646

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668

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672 A.D. Original idea conception, designed the research, performed the research, analysed the  
673 data, wrote and edited the paper; A.H. and N.S. Designed the research, performed the  
674 research, analysed the data, wrote and edited the paper; M.B. and I.P.S. Performed the  
675 research and contributed ideas to perform it, analysed the data, wrote and edited the paper;  
676 A.C., H.F., D.S., J.D. P.F. Performed the research and analysed the data

677

678 **Declaration of Interests**

679 No financial, personal, or professional interests have influenced the work.

680

681 **Data availability**

682 The data integral to the paper (fully documented LCMS/MS analysis) is available through  
683 <https://royalholloway.figshare.com/>. The DOI is [10.17637/rh.13079153](https://doi.org/10.17637/rh.13079153).

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906 **TABLES**

907

908 **Table 1.** A priori defined contrasts used as filtering conditions for the discovery of metabolites  
909 inhibiting breast cell cancer growth.

Treatment contrast	Cell background	Set	Number of metabolites	Overlap	FOIs
Col <i>gl1</i> +MeJA > Col <i>gl1</i> -MeJA	T-47D	A1	236	73	41 <sup>a</sup>
Col <i>gl1</i> +MeJA > Col <i>gl1</i> -MeJA	No cell control	A2	134		
Col <i>gl1</i> +MeJA > <i>coi1</i> -16B +MeJA	T-47D	B1	427	262	
Col <i>gl1</i> +MeJA > <i>coi1</i> -16B +MeJA	No cell control	B2	341	1040	
Col <i>gl1</i> -MeJA = <i>coi1</i> -16B -MeJA	T-47D	C1*	1188		
Col <i>gl1</i> -MeJA = <i>coi1</i> -16B -MeJA	No cell control	C2*	1251		
<i>coi1</i> -16B +MeJA > <i>coi1</i> -16B -MeJA	T-47D	D1	110	30	10 <sup>b</sup>
<i>coi1</i> -16B +MeJA > <i>coi1</i> -16B -MeJA	No cell control	D2	78		
<i>coi1</i> -16B +MeJA > Col <i>gl1</i> +MeJA	T-47D	E1	345	207	
<i>coi1</i> -16B +MeJA > Col <i>gl1</i> +MeJA	No cell control	E2	268		
Col <i>gl1</i> -MeJA +T-47D > Col <i>gl1</i> -MeJA -T-47D		F1	264	187	117 <sup>c</sup>
Col <i>gl1</i> +MeJA +T-47D > Col <i>gl1</i> +MeJA -T-47D		F2	292		
<i>coi1</i> -16B -MeJA +T-47D > <i>coi1</i> -16B -MeJA -T-47D		F3	251	192	
<i>coi1</i> -16B +MeJA +T-47D > <i>coi1</i> -16B +MeJA -T-47D		F4	277		
Col <i>gl1</i> -MeJA +T-47D < Col <i>gl1</i> -MeJA -T-47D		G1	298	141	98 <sup>d</sup>
Col <i>gl1</i> +MeJA +T-47D < Col <i>gl1</i> +MeJA -T-47D		G2	250		
<i>coi1</i> -16B -MeJA +T-47D < <i>coi1</i> -16B -MeJA -T-47D		G3	350	247	
<i>coi1</i> -16B +MeJA +T-47D < <i>coi1</i> -16B +MeJA -T-47D		G4	292		

910 \*Metabolites in Sets C1 and C2 met the condition if metabolite abundances were not significantly  
911 different at P=0.05. <sup>a</sup> *COI1*-dependent MeJA-induced in Col *gl1*; <sup>b</sup> MeJA-induced in *coi1*-16B; <sup>c</sup> more  
912 abundant in T-47D background (end- or by-products of T-47D metabolism); <sup>d</sup> less abundant or absent  
913 in T-47D background (metabolised cancer cell media).

914

915 **FIGURE LEGENDS**

916

917 **Figure 1. The effects of MeJA treatment or co-culture of Arabidopsis JA mutants on**  
918 **human breast cancer cells T-47D and MDA-MB-361 and on non-tumorigenic MCF-10A**

919 **A.** Effect of methyljasmonate (MeJA) treatment on the growth of cancer T-47D, MDA-MB-361  
920 or non-tumourigenic MCF-10A cell lines. The data quantified by 3-(4,5-dimethylthiazol-2-yl)-  
921 2,5-diphenyltetrazolium bromide (MTT) assay are presented as are presented as relative cell  
922 number (RCN) compared to the vehicle (ethanol) control (-).

923 **B.** Plants were grown vertically *in vitro* and photographed at 10 days after sowing (DAS) before  
924 transferring to plates containing media +/- 50  $\mu$ M MeJA. Plants treated (+) and untreated (-)  
925 were photographed again after 24 hours of treatment at 11 DAS to visualise the rosette  
926 phenotype. Scale bar = 5 mm.

927 **C.** At 11 DAS three 3mm leaf disks were excised and co-incubated in three or four replicate  
928 wells with cancer or non-tumourigenic cells for 72 hours after which an MTT assay was  
929 performed.

930 **D.** Effect of the co-incubation of MeJA treated (+) and untreated (-) plant leaf explants from  
931 wild-type background Col *gl1* or jasmonic acid (JA) mutants *coi1-16B*, *aos*, *COV99* and *cev1*  
932 on the growth of cancer T-47D, MDA-MB-361 or non-tumourigenic MCF-10A cell lines. The  
933 data quantified by MTT assay are presented as RCN of viable cells as a % compared to the  
934 growth control.

935 A and C. Bars correspond to the mean of at least three independent experiments (error bars  
936 denote the standard error of the mean). The number of asterisks denote significance values  
937 against the untreated control (-) of each genotype using two tailed t-test with one asterisk being  
938 significant ( $P < 0.05$ ), two being highly significant ( $P < 0.01$ ), three being very highly significant  
939 ( $P < 0.001$ ) and ns being not significant. For detailed P- values see Supplemental Table II.

940

941 **Figure 2. Comparative metabolite analysis of Arabidopsis plant leaf disk bioassay**

942 **A.** Principal component analysis showing discrimination of T-47D and no cells background,  
943 Arabidopsis wild-type (WT) and *coi1-16B* plant leaf disks, and methyljasmonate (MeJA)  
944 treatment regime, based on metabolite profiles.

945 **B.** The heat map shows normalized abundances of all detected chemical features. Samples  
946 and chemical features (putative metabolites) were clustered based on the Euclidean distances  
947 of their normalized metabolite abundances using Ward's (clustering) algorithm. Sets A1, A2,  
948 B1, B2, C1, and C2 show the chemical features that match (red bars) the conditions of the  
949 filtering analysis (A: Col *gl1* +MeJA > Col *gl1* -MeJA; B: Col *gl1* +MeJA > *coi1-16B* +MeJA; C:

950 Col *gl1* -MeJA = *coi1*-16B -MeJA; 1: T-47D background; 2: no cell controls; P=0.05; see Table  
951 1 for details and number of metabolites meeting the conditions). C. Venn diagram of common  
952 features matching filtering conditions in T-47D background, where 146 features of interest  
953 were identified, most of which are part of the two highlighted clusters cl1 and cl2 in the heat  
954 map. R scripts used to analyse data and to generate the figures are provided as Supplemental  
955 data.

956

957 **Figure 3. Abundance of selected Arabidopsis metabolites identified through MS/MS and**  
958 **database searches.** Abundance data (from positive or negative ion mode) correspond to the  
959 peak areas (not normalised) determined by analysis with ProFinder and Mass Profiler  
960 Professional. The compounds abundance was compared in samples containing Arabidopsis  
961 WT (wild-type, Col *gl1*) or mutant *coi1*-16B in presence or absence of T-47D cells and/or  
962 methyljasmonate (MeJA) against media only (m). Umbelliferone was used as an internal  
963 standard. Values denote averages +/- SD (n=2/3).

964

965 **Figure 4. Cell cycle regulators and components of the mTOR signalling pathway are**  
966 **altered in breast cancer cells upon MeJA treatment or incubation with Col *gl1* leaf disks.**  
967 T-47D cells were subjected to 2 mM methyljasmonate (MeJA) or co-incubated with excised  
968 leaf disks of Col *gl1* seedlings treated (+) or untreated (-) with MeJA for 72 hours and protein  
969 levels analysed.

970 A. Western blot detection of Cdc6, Cyclin E1, D1, D3, PCNA, CDK2, p21, p27, in T-47D cells.  
971  $\beta$ -Actin protein levels and Ponceau-S staining were used as to determine equal loading.

972 B. Western blot detection of mTOR, p-TOR Ser2481, p-TOR Ser2448, RICTOR, RAPTOR and  
973 G $\beta$ L in T-47D cells using the indicated antibodies. Samples were harvested after 72 hours  
974 treatment, along with a vehicle treated growth control (mock).  $\beta$ -Actin protein levels and  
975 Ponceau-S staining were used as to determine equal loading.

976

977 **Figure 5. Differential regulation of breast cancer cell growth by MeJA or MeJA-treated**  
978 **leaf disks.**

979 Treatments with methyljasmonate (MeJA) (left) or MeJA-treated leaf disks (right) on breast  
980 cancer cells alter protein levels of different core cell cycle regulators and the mTOR pathway,  
981 resulting in tumour growth inhibition. MeJA treatment induces changes in p21 and Cyclin E1  
982 levels, while inducing the accumulation of the components of both mTORC1 and mTORC2  
983 complexes. In contrast, incubation with MeJA-treated leaf disks affects CDC6, CDK2, Cyclin  
984 D1 and D3, p21 and Cyclin E1, as well inhibits the accumulation of the mTORC2 complex.

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985 Names and 2D structures (PubChem) of compounds discovered through MS/MS are indicated.

986 Red and green shapes indicate accumulation or reduction in protein levels respectively.

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989 **Supporting Information**

990

991 **Methods S1**

992 Further details and information on plant materials, human breast cancer cell lines,  
993 treatment, antibodies and analysis

994 **References to Supplemental Material and Methods**

995 **Supplemental Figures (1-4)**

996 Fig.S1. Methyl jasmonate inhibits cell cycle progression and increases cell death.

997 Fig.S2. Brightfield microscope image of T-47D cells after treatment with 2 mM MeJA  
998 compared to exposure of Colgl1 plant leaf disks.

999 Fig.S3. Screening Arabidopsis metabolism mutants with breast cancer T-47D and non-  
1000 tumorigenic MCF-10A cells

1001 Fig.S4. Transcript analysis of cell cycle marker genes in the human breast cancer cell  
1002 line T-47D upon MeJA treatment or incubation with Col gl1 leaf disks

1003

1004 **Supplemental figure legends**

1005

1006 **Supplemental Tables (I-V)**

1007 Table I. Human QRT-PCR primers used in this study. Excel file

1008 Table II. Inhibition values for Arabidopsis mutants on breast cancer cells. Excel file

1009 Table III. List of 1757 features of interest (FOIs) across all treatments in positive and  
1010 negative ion mode obtained using MassProfiler. Excel file

1011 Table IV. Metabolite features identified. Excel file

1012 Table V. Compound identification information. Excel file

1013

1014 **Notes S1 - R-Script for the analysis reported at Fig 2**

1015 R-script used for comparative metabolite analysis to identify Features of Interest  
1016 (FOIs)

1017