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RESEARCH PAPER

The α-subunit of the heterotrimeric G-protein affects jasmonate responses in *Arabidopsis thaliana*

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Abstract

Heterotrimeric G-proteins have been implicated in having a role in many plant signalling pathways. To understand further the role of G-proteins, a preliminary experiment was performed to assess the impact of the Gα subunit loss-of-function mutation *gpa1-1* on the *Arabidopsis* transcriptome. The analysis indicated that the Gα subunit may play a role in response to jasmonic acid (JA). Consistent with this, Gα mutants showed a reduced response to JA in inhibition of chlorophyll accumulation and root growth, whilst Gα gain-of-function plants overexpressing Gα showed the opposite phenotype. The levels of JA and related compounds were unaffected in the *gpa1-1* mutant, as was autoregulation of the *Allene Oxide Synthase* (*AOS*) gene that encodes a key enzyme for JA biosynthesis. In contrast, further analyses using Gα loss- and gain-of-function *Arabidopsis* lines indicated that Gα positively modulates the expression of the *Vegetative Storage Protein* (*VSP*) gene. This indicates that the Gα subunit regulates a subset of JA-regulated genes defining a branch point in this signalling pathway in *Arabidopsis*. Further analysis of the impact of Gα loss of function upon the JA-regulated transcriptome using *Arabidopsis* full genome arrays indicated that up to 29% of genes that are >2-fold regulated by JA in the wild type are misregulated in the Gα mutant. This supports the observation that a significant proportion of, but not all, JA-regulated gene expression is mediated by Gα.

Key words: *AOS*, *Arabidopsis*, heterotrimeric G-protein, G-protein α-subunit (GPA1), jasmonic acid, *PDF1.2*, *VSP*.

Introduction

Heterotrimeric G-proteins have been identified in a number of plant species and have been implicated in having a role in several plant signalling pathways. Heterotrimeric G-proteins are composed of distinct α, β, and γ subunits that are thought to be activated by seven transmembrane G-protein-coupled receptors (GPCRs). The Gα subunit possesses an intrinsic GTPase activity, and its binding to GTP upon activation leads to its dissociation from the Gβγ complex. An active GTP-bound form of the Gα subunit functions on its own, while Gβ and γ subunits form an obligate heterodimer. This allows both the Gα subunit and Gβγ complex to regulate their respective effectors, thus initiating the downstream signalling cascades (Oldham and Hamm, 2008).

In plants, the basic components of the mammalian G-protein system are found to be conserved; for example, *Arabidopsis* AtGPA1 and AGB1 are 36% and 42% identical at the amino acid level to human Gα and Gβ subunits, respectively (Ma, 1994). The 3D structure deduced from these sequences has indicated that the functionally important regions of G-proteins are also highly conserved in the *Arabidopsis* heterotrimer subunits (Ullah *et al.*, 2003). The *Arabidopsis* genome encodes a simple heterotrimeric G-protein system comprising a single canonical Gα gene,

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AtGPA1 (AGI, 2000; Ma *et al.*, 1990), a Gβ gene, *AGB1* (Weiss *et al.*, 1994), and two Gγ genes, *AGG1* (Mason and Botella, 2000) and *AGG2* (Mason and Botella, 2001). In addition there are reports of a canonical GPCR, *GCR1*, with seven transmembrane domains (Josefsson and Rask, 1997; Plakidou-Dymock *et al.*, 1998; Jones and Assmann, 2004) and a single Regulator of G-protein Signalling (RGS1) protein (Chen *et al.*, 2003) that accelerates G-protein inactivation in *Arabidopsis*.

The lack of redundancy of the G-protein subunit genes is conserved across the plant kingdom, and therefore *Arabidopsis* provides a unique opportunity to investigate the role of G-proteins in a much less complex system than that of animals (Temple and Jones, 2007). Despite the fact that there are only two possible heterotrimeric G-protein complexes in plants, they have been implicated in a wide range of signalling pathways. Pharmacological studies have identified a role for heterotrimeric G-proteins in signalling pathways regulated by a number of phytohormones as well as by biotic and abiotic environmental signals such as pathogens, ozone, and light (Assmann, 2005). Analysis of mutants lacking AGB1 and AtGPA1 have confirmed a role for G-proteins in response to the phytohormones gibberellin (GA) (Ullah *et al.*, 2002), abscisic acid (ABA) (Wang *et al.*, 2001; Ullah *et al.*, 2002; Pandey *et al.*, 2006), auxin (Ullah *et al.*, 2001, 2003), and brassinolides (Chen *et al.*, 2004).

Evidence of G-protein function in plant pathogenesis responses has been demonstrated using the rice *dwarf1* (*d1*) mutant lacking a functional Gα gene (Ashikari *et al.*, 1999; Fujisawa *et al.*, 1999). *d1* mutants were demonstrated to have a reduced hypersensitive response and thus are susceptible to infection by an avirulent form of rice blast fungus, *Magnophorthe grisea* (Suharsono *et al.*, 2002). It has been reported that stomata closure by pathogen-associated molecular patterns (PAMPs) via inhibition of inward K^+ channels requires the Gα subunit in *Arabidopsis* (Zhang, 2008). It has also been demonstrated in *Arabidopsis* that the Gβ subunit is required for resistance to necrotrophic pathogens (Llorente *et al.*, 2005; Trusov *et al.*, 2006).

A transcriptomic analysis has been performed using a Gβ mutant, *agb1-2*, and the Gβ subunit was found to repress a substantial number of auxin-inducible genes in *Arabidopsis* (Ullah *et al.*, 2003). However, to date, transcriptome analysis has not been performed with a Gα mutant. In order to obtain an initial understanding of G-protein regulation of gene expression, a preliminary microarray analysis of the Gα mutant *gpa1-1* was first performed using the Affymetrix (ATH22k) *Arabidopsis* full genome array. Analysis of genes identified in this screening experiment suggested that the Gα subunit has a role in modulating the expression of jasmonic acid (JA)-inducible genes. This was investigated in more detail by examining chlorophyll loss, gene expression, and root growth in response to methyl jasmonate (MeJA). Finally, further transcriptome analyses were undertaken to show that 29% of the JA-regulated genes are misregulated by the loss of the Gα subunit. Possible roles for G-proteins in JA signalling are discussed.

Materials and methods

Plant material and growth conditions

The T-DNA insertion null mutants of GPA1, *gpa1-1* (Ullah *et al.*, 2001) in the Wassilewskija (WS) ecotype background, and *gpa1-3* and *gpa1-4* (Jones *et al.*, 2003) in the Columbia ecotype background, were used. The wGα line in the WS background that inducibly overexpresses wild-type GPA1 protein was also used (Okamoto *et al.*, 2001). Seeds were surface sterilized and sown on plates containing 0.8% (w/v) agar (plant cell culture tested; Sigma-Aldrich, St Louis, MO, USA) and Murashige and Skoog (MS) medium pH 5.7 (Melford, Suffolk, UK). After sowing, the seeds were stratified at 4 °C in darkness for 2 d before transferring to a temperature-controlled tissue culture chamber for 9 d with a 16 h light (70 μmol $m^{-2}s^{-1}$) and 8 h dark day/night cycle, both at 21 °C. Plants used for JA measurements were grown on soil under short-day conditions with 8 h light (140 μmol $m^{-2}s^{-1}$) at 22 °C, 16 h dark at 18 °C of light for 3 weeks before harvesting.

Plants used for northern blot hybridization and chlorophyll measurements were grown as described above except that the seedlings were treated with 50 μM MeJA (jasmonate methyl ester, Sigma-Aldrich) or 70 nM dexamethasone (DEX; Sigma-Aldrich). Plants tested for root growth were sown and grown vertically on half-strength MS agar medium with or without MeJA, and Columbia ecotype seedlings were grown side by side with the coronatine-insensitive *coi1-16* mutant. It was ensured that seeds all germinated at the same time irrespective of the genotype and the presence or absence of MeJA. Chlorophyll was measured and calculated according to the method described by Chory *et al.* (1991).

For the Affymetrix arrays, 10 seedlings of 9-d-old wild type (WS ecotype) and *gpa1-1* were transferred to multiwell plates containing sterile deionized water for 24 h (as the original experiment involved a comparison with ABA treatment which necessitated pre-submergence to control against induction of osmotically regulated genes in response to hypo-osmotic stress induced by transferring seedlings from plates into liquid). For the transcriptome analysis using 3DNA microarray analysis comparing wild-type (Columbia ecotype) and the *gpa1-4* mutant, seedlings were grown for 10 d on agar plates containing half-strength MS medium and then transferred to agar plates containing half-strength MS medium with or without 20 μM MeJA for 6 h. Four biological replicates each consisting of 10 seedlings were harvested for each genotype with and without treatment.

Microarray hybridization

Total RNA was extracted as described below from one biological replicate each of *gpa1-1* and WS wild type. RNA samples for Affytmetrix array were processed by The Nottingham Arabidopsis Stock Centre (NASC; http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=57),

and the normalized CEL files were provided for analysis. Total RNA from four biological replicates for each sample obtained from *gpa1-4* and Columbia wild type were used for Operon 70 mer Arabidopsis microarrays and were processed by the authors in Oxford. Samples were processed according to the manufacturer's instructions (Genisphere, Hatfield, PA, USA) except that 2 μg of total RNA was used for cDNA synthesis using the 3DNA 900 indirect labelling kit and Superscript III (Invitrogen). Arabidopsis full genome microarrays (kindly supplied by Professor David Galbraith, University of Arizona, Tucson, AZ, USA: http://ag.arizona.edu/microarray/deconvolutionver3.0.html) were baked at 80 °C for 30 min and then UV cross-linked at 300 mJ. Slides were pre-hybridized for 20 min at 65 °C in a coupling jar containing 3.5× SSC, 0.1% (w/v) SDS, and 10 mg ml^{-1} bovine serum albumin (BSA), immediately prior to use. Following pre-hybridization, slides were washed for 1 min in distilled water and for 1 min in isopropanol, and then dried using an airbrush. The cDNA was hybridized to the array using SlideBooster SB400 (Advalytix, Munich, Germany) with a power of 27 and a pulse/pause of 3/7 at 55 °C for 16 h. The slides were washed at 55 °C in 2× SSC, 0.2% (w/v) SDS for 10 min followed by room temperature washes of 2× SSC and 0.2× SSC, and dried with an airbrush. The slides were then hybridized with the 3DNA dendrimer capture reagents (Genisphere) for 4 h, washed as before, and scanned using ScanArray Express HT (Perkin Elmer, Waltham, MA, USA) using automatic sensitivity calibration with a maximum signal target ratio of 98%. Dye swap hybridization was made between samples treated with 20 μM MeJA and the control on four slides each for the wild type and *gpa1-4*.

Transcriptome analysis

One replicate each of wild-type and *gpa1-1* Affymetrix array data sets were normalized and 134 genes that were >1.5-fold differentially regulated in *gpa1* compared with the wild type and whose present call was 100% in at least one of the arrays were extracted by DChip ver. 1.3 (Harvard Statistics; http://www. biosun1.harvard.edu/complab/dchip/). The purpose of using a relatively moderate cut-off criterion of 1.5-fold to do this assay was to make it possible to work with a cohort of genes of reasonable size. Median probe intensity was used to normalize the arrays and a permutation-only model was used to obtain model-based expression. Publicly available Affymetrix transcriptome data sets from TAIR Microarray experiments (http://www.arabidopsis.org) from 7-d-old seedlings treated with ABA (ME00333), 1-aminocyclopropane-1-carboxylic acid (ACC; ME00334), brassinolide (ME00335), GA (ME00343), IAA (ME00336), MeJA (ME00337), and zeatin (ME00344) were used to direct the analysis of this gene list further. Genes that were >2-fold regulated after either 30 min, 1 h, or 3 h following application of these plant hormones (with a present call of 100% in all slides) were focused on in the analysis.

Microarray images obtained by 3DNA hybridization were analysed and quantified using BlueFuse (BlueGnome, Cambridge, UK). The data were manually flagged within BlueFuse to remove hybridization artefacts. Data analysis was performed using BASE (Saal, 2002) maintained by the Computational Biology Research Group at the University of Oxford. Cross-channel correction was performed using a 2% setting to account for labelling interactions (Cobbold and Saunders, unpublished). Global median normalization was performed using spots with intensity >200, excluding the 10% most intense and weak probes, and the 10% most increased and decreased fold ratios. The mean fold ratios, Student's *t*-test, and Cyber-T analysis were determined within BASE. The BASE tools were used for cross-channel correction, normalization, and Cyber-T statistical tests (Long *et al.*, 2001). The MIAME compliant data have been submitted to EBI ArrayExpress (www.ebi.ac.uk/arrayexpress) with the accession number: E-MEXP-1822 for public release on publication. The accession numbers of the Array design file (ADF) for these slides are A-MEXP-555 and A-MEXP-1429.

JA measurements

LOX-derived products were analysed as described previously, but with some modifications (Weichert *et al.*, 2002; Göbel *et al.*, 2003). A 1 g aliquot of frozen plant material was added to 20 ml of extraction medium [hexane:2-propanol, 3:2 (v/v), with 0.0025% (v/v) butylated hydroxytoluene] and was immediately homogenized with an Ultra Turrax for 45 s under a stream of argon on ice. The extract was shaken for 10 min and centrifuged at 3200 *g* at 4 °C for 15 min. The clear upper phase was collected, and a 6.7% (w/v) solution of potassium sulphate was added up to a volume of 32.5 ml. After vigorous shaking and centrifugation at 3200 *g* at 4 °C for 10 min, the upper hexane-rich layer that contained oxylipins was subsequently dried under a nitrogen stream. The remaining lipids were re-dissolved in 0.2 ml of methanol and stored under an argon atmosphere at −20 °C until use.

For detection of JA, 12-oxo-phytodienoic acid (OPDA), and dinor-OPDA, compounds were converted to their pentafluorobenzyl esters after purification by reversed phase high-performance liquid chromatography (RP-HPLC) according to Mueller and Brodschelm (1994). The analysis was carried out using a ThermoFinnigan Polaris Q mass selective detector connected to a ThermoFinnigan Trace gas chromatograph equipped with a capillary Rtx-5MS column (15 m×0.25 mm, 0.25 μm coating thickness; Resteck). Helium was used as the carrier gas (1 ml min^{-1}). The temperature gradient was 60 °C for 1 min, 60–180 °C at 25 K min^{-1}, 180–270 °C at 5 K min^{-1}, 270 °C for 1 min, 270–300 °C at 10 K min^{-1}, and 300 °C for 20 min. The pentafluorobenzyl esters were detected by negative chemical ionization with ammonium as the ionization gas. Under these conditions, the retention times of the pentafluorobenzyl esters of JA, OPDA, and dinor-OPDA were 11.7, 20.9, and 18.1 min, respectively. For quantification, the ions *m/z* 209 (JA), 291 (OPDA), and 263 (dinor-OPDA) were used, respectively.

RNA blot analysis

Total RNA was extracted using RNeasy kits (Qiagen, Crawley, UK) according to the manufacturer's protocol, and 10 μg of each RNA sample was used to perform northern hybridization as described previously (Knight *et al.*, 1999). Primers to coding sequences of the genes: *Allene Oxide Synthase* (*AOS*; At5g42650) (forward, 5′-TCCACAAGTCGTGGCTTTAC-3′; reverse, 5′-AATCTCTCCGGCACAAACTC-3′), *PDF1.2b* (At2g26020) (forward, 5′-GTTGATCTCTTAAGCTTAT-3′; reverse, 5′-CCAAGTACCACTTGGCTTC-3′), putative *Thionin* (At1g66100) (forward, 5′-CAAGTGAAATCGTGA-ATGGAG-3′; reverse, 5′-TATATGAAGTATCGAGGATC-T-3′), *Vegetative Storage Protein* 1 (*VSP1*; At5g24780) (forward: 5′-ATGAAAATCCTCTCACTTTC-3′; reverse, 5′-GTGAGATGATGCATGGAAGA-3′), *AtMYC2*/*JIN1*/*ZBF1* (At1g32640) (forward, 5′-ATGACTGATTACCGGC-TACA-3′; reverse, 5′-TCTGCGTCATCGAAACCA-3′), *ERF1* (At3g23240) (forward, 5′- TCAGTCCCCATTCTCC-GGCT-3′; reverse, 5′-TCACCAAGTCCCACTAT-3′), and *GPA1* (At2g26300) (forward, 5′-ATGGCTGCAACTGCAA-TCTC-3′; reverse, 5′-CTAAGATTTCCGTTCAACCAAGA-3′) were used to generate probe DNA by PCR. The β-tubulin probe was prepared from the PCR products using specific primers as described previously (Knight *et al.*, 1999). Probes were labelled with [^{32}P]dCTP using Ready-ToGo probe synthesis beads (Amersham Biosciences) and purified through ProbeQuant G-50 micro columns (Amersham Biosciences).

Results

Transcriptomic analysis of a GPA1 null mutant suggests a role for Gα in JA-dependent gene expression

A role for the Gα subunit and G-proteins in ABA signalling is well documented (Wang *et al.*, 2001; Jones and Assmann, 2004; Pandey and Assmann, 2004; Pandey *et al.*, 2006) and it was decided to test the effect of Gα loss of function on the ABA-responsive transcriptome (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=57). RNA samples were obtained from seedlings treated with or without 100 μM ABA for 3 h. Although 684 genes showed a >2-fold change in expression after ABA treatment in the wild type, >90% of these genes were also regulated >2-fold in *gpa1-1*. In addition, expression of genes including *KIN1*/*2* and *P5CS* in response to ABA was indistinguishable between the wild type and *gpa1-1* by northern blot analysis (data not shown). The lack of a significant effect of the *gpa1-1* mutation on ABA-responsive gene expression in the analysis using whole seedlings may suggest that the role of Gα in ABA-regulated gene expression is tissue specific (e.g. guard cells). Alternatively, as it has been reported that exogenously applied ABA leads to an increase in GPA1 protein detectable by immunoblot analysis (Pandey *et al.*, 2006), it cannot be ruled out that Gα is acting independently of changes in gene expression during the first 3 h of response to ABA.

To exploit further the data set generated, the differences in expression of genes in *gpa1-1* compared with the wild type was examined in the non-ABA-treated seedlings. It was found that 134 genes were >1.5 times differentially expressed (67 down- and 67 up-regulated) in *gpa1-1* compared with the wild-type control in this comparison (for a full list see Supplementary Table S1available at *JXB* online). Since plant G-proteins mediate the responses of several plant hormones (Fujisawa *et al.*, 2001; Perfus-Barbeoch *et al.*, 2004), it was examined whether these 134 genes were enriched in genes previously reported to be regulated by hormones. The Affymetrix transcriptomic data sets from the TAIR Microarray database (http://www.arabidopsis.org) were used for this analysis. By calculating how many of the genes from the list of 134 *gpa1-1*-affected genes were also regulated by each hormone, it was determined whether genes which respond to a particular hormone were more abundant in this list than would be expected by chance (Supplementary Table S2). Using this approach, it was observed that genes regulated by ABA and MeJA were most significantly enriched, by 2.7- and 2.8-fold, respectively. Binomial probability analyses indicated that this enrichment was particularly high for ABA- (probability of FALSE; 1.98E-06) and MeJA-regulated (1.45E-03) genes (Supplementary Table S2). Enrichment for genes regulated by ACC (a precursor of ethylene), brassinolide, GA, IAA, and zeatin was not significant. This comparative transcriptomic analysis of *gpa1-1* and the the wild type consisted only of a single biological replicate. Nevertheless, it suggested a role for the Gα subunit in the response of *Arabidopsis* to MeJA, and consequently further analyses were performed to investigate this.

The Gα subunit regulates MeJA-dependent chlorophyll reduction

In light of the preliminary transcriptomic data that were obtained, the physiological characteristics of Gα mutants in response to MeJA were then investigated. It has been reported that JA and related compounds reduce chlorophyll content when applied exogenously to leaves (Parthier, 1990). Test were first carried out to determine whether Gα mutants were impaired in JA-dependent chlorophyll reduction. Eleven-d-old seedlings were treated with 0 μM or 50 μM MeJA for 3 d and the chlorophyll levels in cotyledons were measured. The ratio of total chlorophyll between MeJA-treated and non-treated cotyledons is shown in Fig. 1A and B. As described by Parthier (1990), MeJA-treated wild-type plants became significantly more yellow, particularly in the cotyledons, and the total chlorophyll was reduced to 33.5% and 19.8% of that of an untreated WS and Columbia ecotype control, respectively. However, *gpa1* mutants retained >40% chlorophyll after MeJA treatment.

Inducible (by DEX) ectopic expression of Gα caused leaves to be paler due to a reduction in chlorophyll compared with control plants (Fig. 1C), while DEX

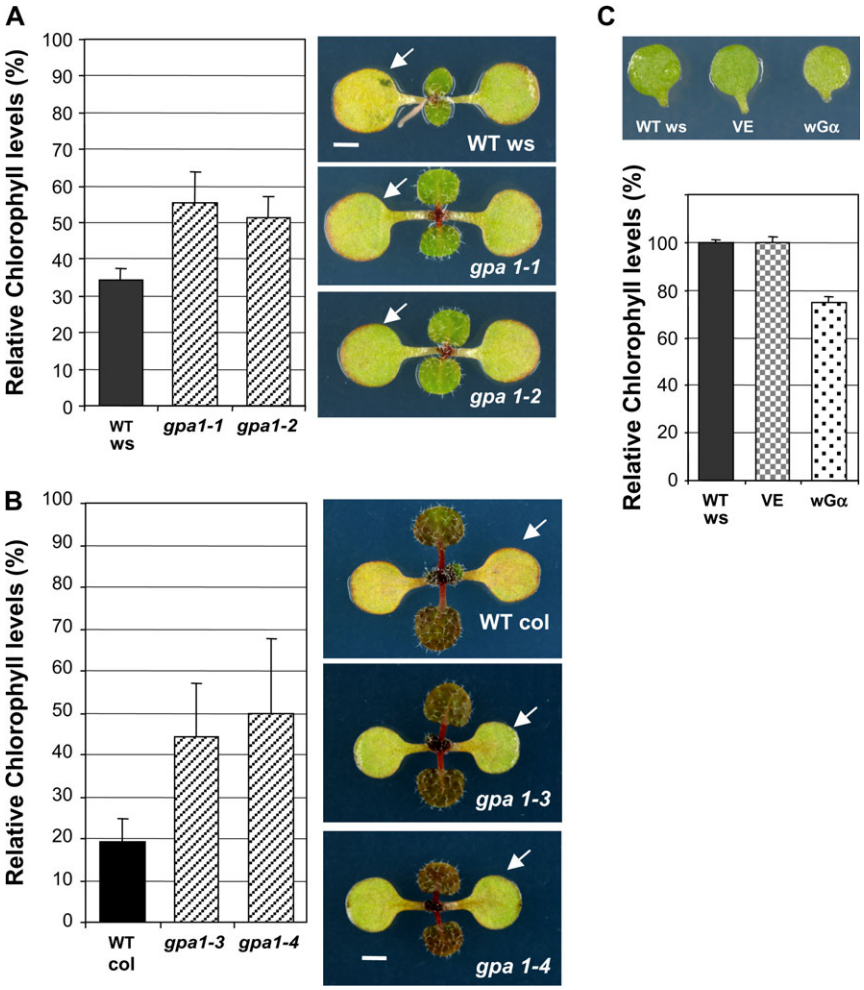

Fig. 1. MeJA-dependent chlorophyll regulation is reduced in *gpa1* mutants. Eleven-day-old plants grown on MS agar plates were treated with either MeJA (A and B) or DEX (C) for a further 3 d. Top views of the seedlings treated with MeJA for 3 d are shown in (A): WT ws, Wassilewskija wild type; VE, empty vector transgenic line; wGα, Gα overexpressor plants; and in (B) Gα mutants, *gpa1-1, gpa1-2*; and WT ws, Wassilewskija wild type. Cotyledons of the representative plants treated with DEX for 3 d are also shown in (C) Gα mutants, *gpa1-3*, *gpa1-4*; and WT col, Columbia wild type. Total chlorophyll was extracted from the cotyledons of these plants and the chlorophyll levels are shown as the ratio between treated and non-treated control plants. Bars represent 1 mm.

treatment did not change the total chlorophyll content in wild-type or in empty vector transformant (VE) control plants. It was previously reported that overexpression of the Gα subunit resulted in reduced expression of both *CAB* and *RBCS* (Okamoto *et al.*, 2001). Whilst this finding cannot be formally attributed to MeJA responses specifically, as this effect occurs in the absence of exogenously applied MeJA, the current results strongly suggest that the Gα subunit has a role in the regulation of chlorophyll levels in response to JA signalling.

Gα subunit regulates JA-dependent root growth inhibition

JA regulation of seedling and root growth (Lorenzo and Solano, 2005) is one of the most well examined phenotypes in *Arabidopsis*. In order to investigate further the role of Gα in JA signalling, JA-dependent root growth inhibition was measured in *gpa1* mutants and a range of JA concentrations that have been used for this assay was tested (Staswick *et al.*, 1992). The results showed that *gpa1* mutants are significantly insensitive to 1 μM MeJA compared with the wild type (Fig. 2A, B). Staswick and colleagues demonstrated that at 1 μM MeJA Columbia ecotype roots showed 50% growth inhibition (Staswick *et al.*, 1992), which is consistent with the present assay (Fig. 2A). The difference in root length between the wild type and *gpa1* mutants was statistically significant in all four alleles. It was then tested if overexpression of Gα is also sufficient for the inhibition of root growth. The result showed that the root growth was >50% inhibited by Gα overexpression (Fig. 2C). These data suggest that Gα has a positive role in response of roots to JA.

The gpa1-1 *mutant is impaired in JA-inducible gene expression*

The Affymetrix transcriptome data indicated that JA regulation of gene expression may be affected in *gpa1*

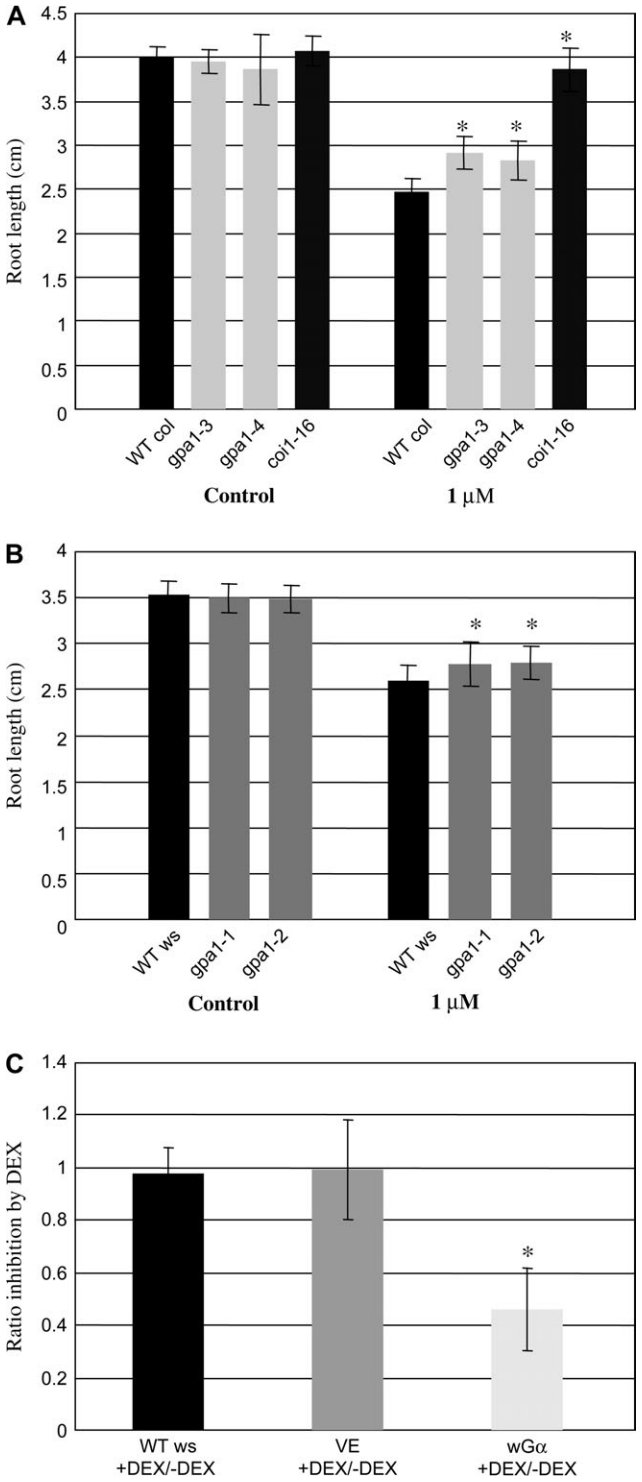

Fig. 2. MeJA-dependent root growth regulation is reduced in *gpa1* mutants. Seedlings were grown on half-strength MS agar medium containing the indicated amount of MeJA for 11 d and the root lengths were measured. (A) Mean root lengths of *gpa1-3* and *1-4* as well as the WT (wild-type) Columbia ecotype and the coronatine-insensitive 1 (*coi1-16*) mutant that is insensitive to root growth inhibition by exogenous MeJA are shown. (B) Mean root length of *gpa1-1* and *1-2* in the Wassilewskija (WS) background is shown with the WS wild type. (C) Inhibition of root growth by over-expression of Gα is shown along with the WS wild-type control and the vector transformant control. The asterisk represents a significant difference in mean root length between the mutant line and the respective wild type ($P < 0.05$) by Student's *t*-test for one-tailed assuming equal variance. Error bars represent the SD.

mutants. The regulation of one of the best characterized MeJA-inducible genes, *VSP1*, was therefore examined (although it was not on the Affymetrix arrays that was used). Ther results show that *VSP1* was induced by MeJA in wild-type plants, with the highest accumulation detected 4–6 h after MeJA application. Significantly, induction of *VSP1* transcript was severely affected in the *gpa1-1* mutant, with only very low levels of expression detectable (Fig. 3). It should be noted that the probe used for *VSP1* has ~90% sequence identity to *VSP2* and it cannot be ruled out that the combined expression of both genes is being measured. However, *VSP2* is less sensitive than *VSP1* to JA induction and it is therefore believed that *VSP1* expression is primarily being measured.

Although they are not solely regulated by JA, the basal expression levels of several *PDF1.2* genes were found to be expressed relatively more strongly in *gpa1-1* than in wild-type plants in the transcriptome data (Supplementary Table S2at JXB online). *PDF1.2b* gene expression was therefore examined in *gpa1-1*. *PDF1.2b* was induced by MeJA in wild-type plants, with expression peaking at ~8 h (Fig. 3), but MeJA-dependent *PDF1.2b* expression was detectable much earlier in *gpa1*.

Similarly to *PDF1.2b*, expression of the putative *Thionin* gene (At1g66100) was found to be relatively low in *gpa1* compared with the wild-type in the transcriptome data. Thionins are small cysteine-rich proteins with antimicrobial activity (Pelegrini and Franco, 2005) and are known to be induced by MeJA. The putative Thionin predicted amino acid sequence shows a sequence identity of 59% and 57% to Thi2.2 (At5g36910) and Thi2.1 (At1g72260) genes, respectively. In order to verify that *gpa1-1* is impaired in MeJA induction of this putative *Thionin*, expression of this gene was also analysed by RNA blot analysis. The result indicated that the putative *Thionin* gene is inducible by MeJA in wild-type plants and that the expression peaks at ~6 h. However, *Thionin* gene expression was below the level of detection by northern analysis in the *gpa1-1* mutant even 6 h after MeJA application (Fig. 3). Together these data suggest that the loss of the Gα subunit affects JA-responsive gene expression.

The gpa1-1 *mutant retains wild-type levels of JA and related precursors*

One possible explanation for the enrichment of JA-regulated genes among the *gpa1-1*-affected genes is that the *gpa1-1* mutant might have altered levels of endogenous JA. In order to determine if this was the case, levels of endogenous free JA and the related precursors, OPDA and dinor-OPDA, were measured in 3-week-old rosette leaves of the wild type and *gpa1-1*. There were no significant differences in any of these JA-related compounds in wild-type

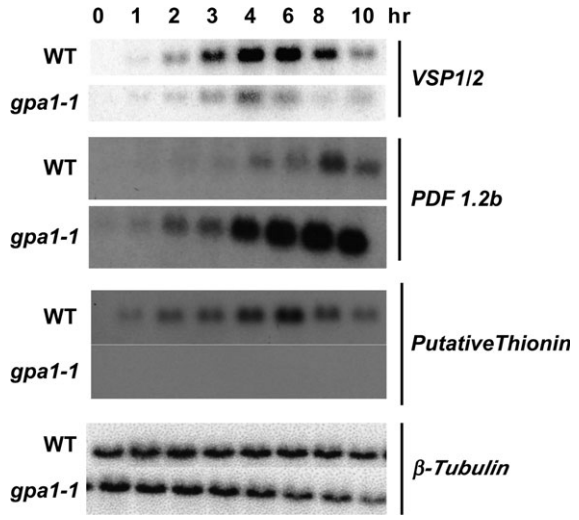

Fig. 3. MeJA-inducible genes are misregulated in the *gpa1* mutant. The MeJA induction time courses of JA-inducible genes were examined using 10-d-old wild-type (WT) and *gpa1-1.* The expression of MeJA-inducible genes, *VSP1/2*, *PDF1.2b*, and putative *Thionin* in *gpa1-1* and the wild type after incubation with 50 μM MeJA for the indicated time is shown. A 10 μg aliquot of total RNA was loaded on each lane and β-*tubulin* was used as a loading control.

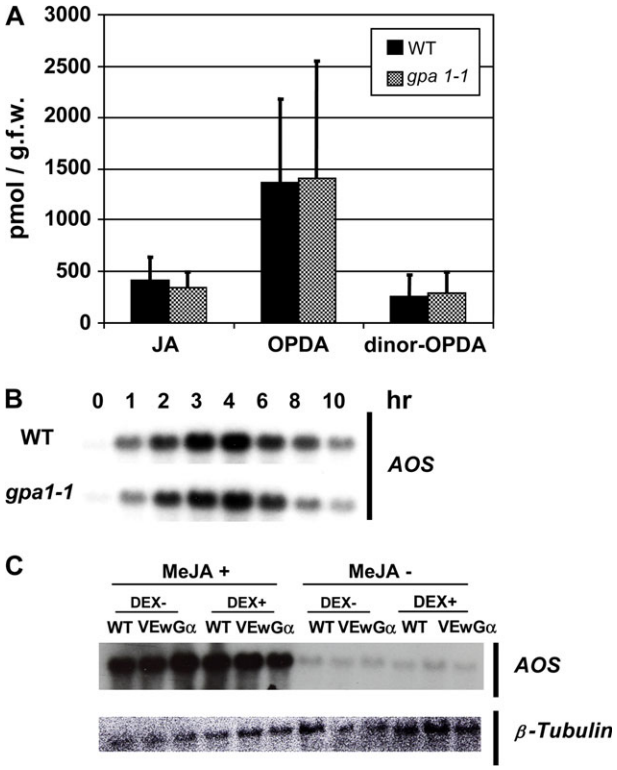

Fig. 4. Gα does not affect endogenous JA levels. (A) JA and its precursors OPDA and dinor-OPDA contents were measured using 3-week-old rosette leaves of WS wild type and *gpa1-1.* Three leaves were harvested from each plant and averages of 11 independent measurements are shown. Error bars represent the SDs. (B) The MeJA induction time course of a JA biosynthesis gene, *AOS*, was examined using 10-d-old wild-type (WT) and *gpa1-1* treated with 50 μM MeJA for the indicated times. The loading control is the same as in Fig. 3. (C) *AOS* gene expression was examined with or without MeJA and Gα overexpression. Eleven-day-old seedlings were treated first with or without DEX for 3 d and were incubated with or without 50 μM MeJA for 6 h. WT, Wassilewskija wild-type control and transgenic plants; VE, vector only transformant; wGα, transgenic plant inducibly overexpressing the Gα subunit. β-*Tubulin* was used as a loading control.

and *gpa1-1* plants (Fig. 4A). Although the data were obtained from adult leaf tissue and not seedlings, they demonstrate that the *gpa1-1* mutant does not show gross effects on JA biosynthesis.

The JA biosynthesis positive feedback pathway is intact in gpa1

JA biosynthesis is autoregulated, and exogenous application of MeJA induces genes involved in JA biosynthesis such as *AOS* (Stenzel *et al.*, 2003). In order to determine if the positive feedback mechanism of JA biosynthesis pathways was affected in Gα mutants, *AOS* induction in response to exogenous application of MeJA was examined in wild-type and *gpa1-1* mutant plants. As shown in Fig. 4B, MeJA induction of *AOS* is intact in the *gpa1-1* mutant and is indistinguishable from that in the wild type.

To confirm further that Gα is not modulating the JA biosynthesis pathway, *AOS* expression in the transgenic line inducibly expressing the Gα subunit (Okamoto *et al.*, 2001) was analysed in the presence and absence of MeJA. *AOS* expression was not influenced by the overexpression of the Gα subunit under either set of conditions (Fig. 4C), indicating that the gain of functional Gα subunit does not have any effect on JA biosynthesis. These results indicated that the altered basal expression of JA-regulated genes in *gpa1* mutants is most likely to have resulted from an alteration in the sensing of or response to JA.

Expression of ERF1 *and* AtMYC2/JIN1/ZBF1 *is not affected in* gpa1

PDF1.2 expression is positively regulated via the ethylene response factor 1, *ERF1*, overexpression of which is sufficient to increase *PDF1.2* expression (Anderson *et al.*, 2004). The exaggerated expression of *PDF1.2* in *gpa1* mutants could therefore have been due to overexpression of *ERF1*. Thus, the expression of *ERF1* in *gpa1* was investigated. The level of *ERF1* hybridization signal on the *gpa1-1* Affymetrix array was similar to that of the wild type. This was confirmed by northern analysis: the basal level of expression as well as MeJA induction of *ERF1* in the *gpa1-1* mutant was indistinguishable from that of the wild type (Supplementary Fig. S1 at *JXB* online). These data indicate that increased *PDF1.2* expression in *gpa1* mutants is

therefore unlikely to be caused by misregulation of *ERF1* transcription factor gene expression.

Co-overexpression of AtMYC2/JIN1/ZBF1 suppresses the induction of *PDF1.2* by ERF1, and AtMYC2/JIN1/ZBF1 has been suggested to act epistatically on ERF1 activation of *PDF1.2* (Anderson *et al.*, 2004). Conversely, mutations in *AtMYC2/JIN1/ZBF1* result in increased expression of *PDF1.2* (Lorenzo *et al.*, 2004) and reduced expression of *VSP1* (Berger *et al.*, 1996), leading to the proposal that AtMYC2/JIN1/ZBF1 positively induces *VSP1* while it negatively regulates *PDF1.2*. The expression of *VSP1/2* and *PDF1.2* in *gpa1* is similar to that in the *atmyc1/jin1* mutant and it was therefore possible that AtMYC2/JIN1/ZBF1 transcription factor gene expression may be reduced in *gpa1*. However, this was not the case and, as for *ERF1*, basal expression as well as MeJA-dependent induction of *AtMYC2/JIN1/ZBF1* in the *gpa1-1* mutant was indistinguishable from that of the wild type (Supplementary Fig. S1 at *JXB* online).

Gα overexpression affects transcription of VSP1/2 *but does not affect that of* AtMYC2/JIN1/ZBF1

As described above, the *gpa1-1* mutant and *jin1* mutants lacking AtMYC2/JIN1/ZBF1 show very similar patterns of expression with respect to regulation of *VSP1* and *PDF1.2* genes. However, unlike *jin1* mutants, expression of *AtMYC2/JIN1/ZBF1* is indistinguishable from the wild type in *gpa1-1*. It is therefore possible that Gα regulates *VSP1/2* independently of *AtMYC2/JIN1/ZBF1* gene expression. If this is the case, overexpression of the Gα subunit should not affect *AtMYC2/JIN1/ZBF1* gene expression but would still be sufficient for the expression of *VSP1/2*. Gα-overexpressing plants accumulated a significantly increased level of *VSP1/2* compared with the non-induced control in the absence of MeJA (Fig. 5A). These data show that Gα overexpression is sufficient for induction of *VSP1/2* expression. Significantly though, Gα overexpression did not induce the accumulation of *AtMYC2/JIN1/ZBF1* (Fig. 5A). It should also be noted that *VSP1/2* accumulation was not detectable when the DEX-treated Gα plants were kept in darkness (Fig. 5A), even when Gα overexpression was induced to ~3 times higher levels than in the light (Fig. 5C). This shows that overexpression of the Gα subunit was sufficient to induce *VSP1/2* expression in the light but not in darkness, and indicates that induction of *VSP1/2* also requires an additional light input.

Gα overexpression is sufficient for the transcription of PDF1.2 *and* ERF1

In order to examine further the role of Gα in the regulation of the *PDF1.2* gene, the expression of *PDF1.2* in Gα-overexpressing plants was also tested. Figure 5B shows that Gα overexpression increases *PDF1.2* in the absence of MeJA. Significantly, Gα-overexpressing plants also had higher accumulation of *ERF1* compared with control plants. These data suggest that the accumulation of *PDF1.2*

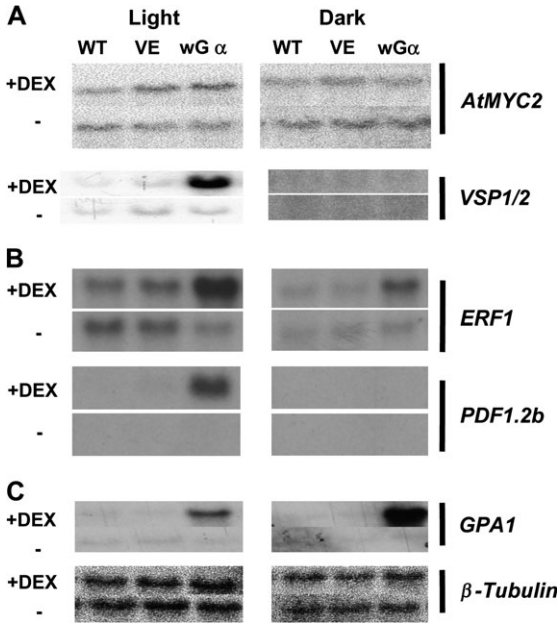

Fig. 5. Gα overexpression is sufficient for the induction of *VSP1* and *PDF1.2* genes. Eleven-day-old WS, wild-type control and transgenic plants; VE, vector only transformant; and wGα, transgenic plant inducibly overexpressing the Gα subunit were treated with or without DEX under light and in complete darkness for 3 d. A 10 μg aliquot of total RNA was loaded on each lane and the same blot was probed with (A) *AtMYC2/JIN1/ZBF1* and *VSP1/2*, (B) *ERF1* and *PDF1.2*, and (C) *GPA1* and β-*tubulin* as a loading control.

transcripts in Gα-overexpressing plants is likely to be the result of the accumulation of *ERF1*.

In addition to this, as was seen for the *VSP1/2* gene, *PDF1.2* gene induction by Gα overexpression was only detectable when the plants were incubated in the light. In the case of *ERF1* gene expression, a moderate induction was consistently detected following Gα overexpression in the absence of light; however, the induction of *ERF1* was higher in the light than in the dark.

Comparative transcriptome analysis of gpa1-4 *and the wild type*

The preliminary microarray experiment using 22k Affymetrix arrays was performed with the wild type and the *gpa1-1* mutant in the WS background. It should be noted that the Affymetrix 22k arrays are designed using the Columbia accession genome sequence. In order to gain further understanding of the global consequence of the loss of Gα function on gene expression, the transcriptomes of 10-d-old wild-type Columbia ecotype and *gpa1-4* mutant seedlings were investigated in a biologically replicated and quantitative microarray comparison. Briefly, seedlings of each biological replicate were treated with 0 μM or 20 μM MeJA for 6 h. Operon *Arabidopsis* full genome microarrays were used to assess four biological replicates of each genotype. Each slide compared the transcriptomes of MeJA-treated and untreated seedlings of either the wild type or *gpa1-4*.

A total of 822 genes were identified that were >2-fold regulated by MeJA in wild-type seedlings. This statistically significant (*P*-values <0.01) cohort of genes are shown in Supplementary Table S3 at *JXB* online. Some genes were detected several times due to multiplication of the probes. Of these 822 genes, 200 were <2-fold regulated in *gpa1-4* (Cluster I; Fig. 6A). Of the remaining 622 genes, 527 genes were >2-fold changed in response to MeJA (P <0.01) in the *gpa1-4* mutant. In addition, 43 genes in this group showed a change in response to MeJA that was more than twice that observed in the wild type (Cluster IIa and b; Fig. 6A, i.e. the direction of change was the same but was more exaggerated in the mutant. Taken together, 243 out of 822 genes (29%) regulated by MeJA in wild-type seedlings were differentially regulated in *gpa1-4*.

The gene set (822 genes) was then compared with the gene set identified as >2-fold regulated by MeJA (760 genes) from Affymetrix array data available from the NASC [http://www.arabidopsis.org (ME00337)], in order to gain cross-platform validation of these observations. Although the biological materials, experimental conditions, and the microarray platforms differed, 247 genes were common to both experiments and data types. The probability of an overlap between randomly chosen sets of 822 genes and 760 genes on custom and 22k Affymetrix arrays, respectively, is <0.1%, meaning that no overlapping genes would be expected between randomly chosen gene sets of these sizes. Given that both arrays are biologically replicated and the differential expression of these genes by MeJA is statistically significant, the 30% overlap observed is meaningful.

In this group of 247 genes (Supplementary Table S4 at *JXB* online), 31 genes were <2-fold regulated in *gpa1-4* (Cluster I; Fig. 6C). Of the remaining 213 genes, 194 were also >2-fold regulated by MeJA (P <0.01) in *gpa1-4*. However, 17 genes (Cluster IIa and b; Fig. 6C) in this group of 194 genes showed a fold regulation that was twice that of the wild type. Together, these analyses show that the regulation of 48 out of 247 (19%) MeJA-regulated genes common to both arrays was misregulated in *gpa1-4*. These data therefore indicate that a loss of the Gα subunit affects MeJA-regulated gene expression.

Discussion

The results presented here provide evidence that the *Arabidopsis* heterotrimeric Gα subunit has a role in modulating plant responses to JA. The Gα-deficient *gpa1* mutant showed an altered response to JA regulation of gene expression whether assessed by the Affymetrix or Operon arrays, and in subsequent northern analysis of JA-responsive genes such as *VSP1/2* and *PDF1.2*. In addition, loss of Gα also affected JA regulation of root growth and chlorophyll accumulation. In contrast, Gα had no effect on JA biosynthesis or the regulation of JA biosynthesis genes, suggesting that the Gα subunit functions in a distinct branch of the JA signalling pathway in *Arabidopsis*.

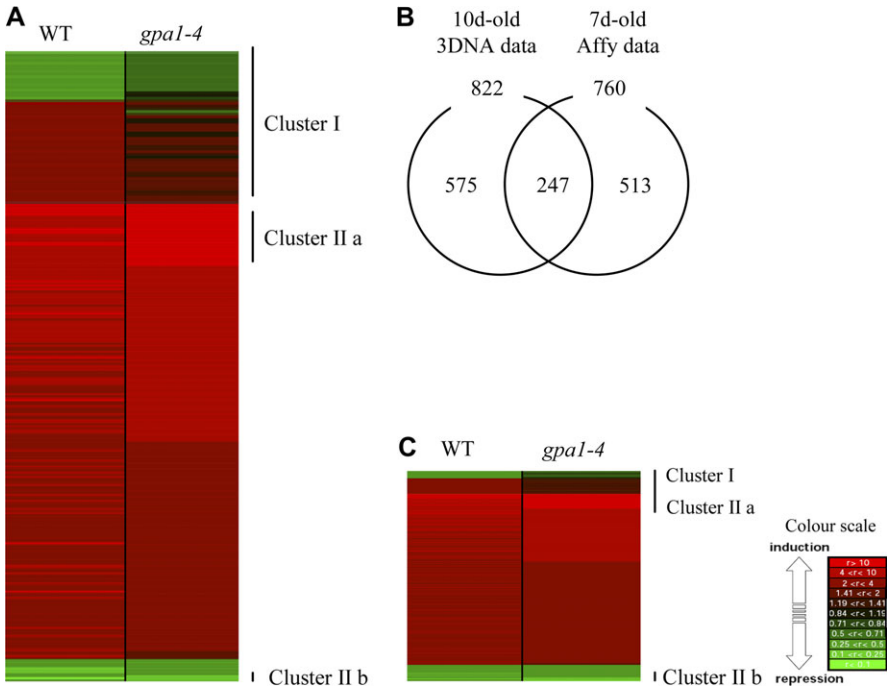

Fig. 6. Loss-of-function Gα has a significant impact on JA regulation of the transcriptome. (A) JA responsiveness in *gpa1-4* was analysed in 822 genes that are >2-fold regulated by MeJA in the wild type. (B) Venn diagram showing the overlap of the 822 genes that were found to be up-regulated by JA in the custom arrays using 10-d-old seedlings, and the 760 MeJA-regulated genes in Affymetrix arrays using 7-d-old seedlings. (C) JA responsiveness in *gpa1-4* was analysed in 247 genes that are commonly regulated by MeJA in the custom arrays and the publicly available Affymetrix arrays. The colour scale shows the fold regulation of the genes.

JA signalling is modulated by Gα

Gα loss- and gain-of-function plants show an opposite and complementary response to exogenous application of JA for induction of both chlorophyll accumulation (Fig. 1) and *VSP1/2* gene expression (Figs 3, 5). It should be noted that the *GPA1* gene is not induced by MeJA in the Affymetrix array performed by Goda and co-workers (ME00337) and in the present custom arrays (Tables S3, S4 at *JXB* online). These results therefore suggest that Gα is positively regulating chlorophyll accumulation and *VSP1/2* gene expression in response to JA. The former result is consistent with the previous observation that *CAB* and *RBCS* gene expression was reduced following Gα overexpression (Okamoto *et al.*, 2001) and that JA induces expression of the chlorophyllase gene *CLH1* in *Arabidopsis* (Tsuchiya *et al.*, 1999), which results in a reduction of the chlorophyll content of the plants (Parthier, 1990). It is possible that JA acts directly as a ligand for heterotrimeric G-protein signalling in *Arabidopsis*, and this should be tested in the future.

Interestingly, overexpression of the Gα subunit was sufficient for induction of the *VSP1/2* gene in the light but not in the dark (Fig. 5A). MeJA induction of *VSP1* gene expression in *Arabidopsis* is induced in the presence of light, but not in the dark (Berger *et al.*, 1995). At least some JA and G-protein signalling may therefore require factors only present when plants are exposed to light. JA biosynthesis genes such as *AOS* and *JAR1* in rice (Haga and Iino, 2004; Riemann *et al.*, 2008) and *JAR1*/*FIN219* in *Arabidopsis* (Hsieh *et al.*, 2000; Chen *et al.*, 2007) have been implicated in light signalling. Additionally, the JA signalling mutant *coi1* shows a number of light-insensitive phenotypes (Robson, Okamoto, and Turner, unpublished results). These data suggest that JA and light signalling are operating closely together and Gα may play a role at the interface of these two signalling pathways. Such a scenario may account for previous observations that Gα is involved in light signalling responses (Okamoto *et al.*, 2001).

An investigation was also carried out to determine if overexpression of Gα is sufficient to induce the putative *Thionin* gene as it is for *VSP1/2*. However, in the absence of MeJA, expression of this gene was not detectable with Gα overexpression alone (data not shown). This may be due to a relatively low expression level of the putative *Thionin* gene compared with *VSP1/2*. It is also worth noting that unlike the *VSP1* gene, the putative *Thionin* promoter does not have a AtMYC2/JIN1/ZBF1 *cis*-binding consensus and therefore this gene is likely to be regulated by Gα in a manner different from *VSP1/2*.

In contrast to *VSP1/2* gene expression and chlorophyll accumulation, the role of Gα in the regulation of *PDF1.2* may be more complex as both the gain- and loss-of-function Gα resulted in hyperaccumulation of *PDF1.2* (Figs 3, 5B). In the case of gain-of-function Gα analysis, the gene encoding the ERF1 transcription factor, for which there is a *cis*-binding consensus sequence present in the *PDF1.2b* promoter, accumulated in the Gα-overexpressing plants without application of exogenous MeJA (Fig. 5B). It is therefore possible that accumulation of *ERF1* is the cause of the increase in *PDF1.2* expression. In loss-of-function Gα, *PDF1.2* gene expression increased without significant accumulation of *ERF1*. Therefore, it is possible that the *PDF1.2* accumulation observed in these two results was mediated by two different pathways.

It has been shown that simultaneous application of ethylene reduces *VSP1* induction by JA (Tuominen *et al.*, 2004), whilst ethylene and JA acts synergistically on *PDF1.2* expression (Penninckx *et al.*, 1998; Ellis and Turner, 2001). The loss-of-function Gα phenotype may have been the result of increased activation of ethylene signalling. However, this is less likely, as the level of *ERF1* transcript that would be expected to be induced in response to ethylene signalling was found to be indistinguishable in *gpa1* mutants and the wild type (Supplementary Fig. S1 at *JXB* online). In contrast to the loss-of-function *gpa1-1* mutant, it is possible that Gα overexpression does indeed lead to the induction of both *ERF1* and *PDF1.2* and therefore may be affected in ethylene signalling. It has been shown that Gβ mutants show hypersensitivity to an ethylene precursor, ACC, with respect to root growth inhibition in darkness (Pandey *et al.*, 2008). This suggests that Gβ may be suppressing ethylene signalling. However, it is also possible that Gα may be acting positively in ethylene signalling, and the gain-of-function result suggests the latter. Despite the complex nature of regulation, these data provided further evidence for a role for Gα in the regulation of gene expression by JA.

A possible regulatory role for G-proteins on AtMYC2/JIN1/ZBF1

Arabidopsis G-proteins work in a wide range of signalling pathways regulated by phytohormones such as GA, ABA, auxin, D-glucose (Chen and Jones, 2004), and brassinolides, as well as by biotic and abiotic environmental signals such as pathogenesis, ozone (Joo *et al.*, 2005), and light (Okamoto *et al.*, 2001). The most likely scenario is that G-proteins are regulating a small number of factors that are common to these signalling pathways. AtMYC2/JIN1/ZBF1 is a common transcription factor not only for light (Yadav *et al.*, 2005) but also for ABA (Abe *et al.*, 2003) and JA (Boter *et al.*, 2004; Lorenzo *et al.*, 2004) signalling pathways in *Arabidopsis*. The present data show that it is possible that Gα is regulating the activity of AtMYC2/JIN1/ZBF1, thus providing a potential mechanism for its involvement in light, ABA, and JA signalling pathways. Future work should be directed at testing if Gα overexpression in a *jin1* mutant background has consequences for *VSP1/2* gene expression and/or the chlorophyll accumulation phenotype.

Supplementary data

Fig. S1. *AtMYC2*/*JIN1*/*ZBF1* and *ERF1* induction by MeJA in the *gpa1-1* mutant and the wild type.

Table S1. The list of 134 *gpa1-1* differentially-regulated genes in the Affymetrix array.

Table S2. Affymetrix array analysis of 134 *gpa1-1* differentially regulated and hormone-regulated genes.

Table S3. The list of 822 genes that were more than 2-fold regulated by MeJA in the wild type with *P*-values of <0.01

Table S4. The list of 247 genes that were common to 10- and 7-d-old seedlings induced by JA.

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