

RESEARCH ARTICLE

# The Arabidopsis KH-Domain RNA-Binding Protein ESR1 Functions in Components of Jasmonate Signalling, Unlinking Growth Restraint and Resistance to Stress

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## Abstract

Glutathione S-transferases (GSTs) play important roles in the protection of cells against toxins and oxidative damage where one Arabidopsis member, *GSTF8*, has become a commonly used marker gene for early stress and defense responses. A *GSTF8* promoter fragment fused to the *luciferase* reporter gene was used in a forward genetic screen for Arabidopsis mutants with up-regulated *GSTF8* promoter activity. This identified the *esr1-1* (*enhanced stress response 1*) mutant which also conferred increased resistance to the fungal pathogen *Fusarium oxysporum*. Through positional cloning, the *ESR1* gene was found to encode a KH-domain containing RNA-binding protein (At5g53060). Whole transcriptome sequencing of *esr1-1* identified altered expression of genes involved in responses to biotic and abiotic stimuli, hormone signaling pathways and developmental processes. In particular was an overall significant enrichment for jasmonic acid (JA) mediated processes in the *esr1-1* down-regulated dataset. A subset of these genes were tested for MeJA inducibility and we found the expression of some but not all were reduced in *esr1-1*. The *esr1-1* mutant was not impaired in other aspects of JA-signalling such as JA-sensitivity or development, suggesting ESR1 functions in specific components of the JA-signaling pathway. Examination of salicylic acid (SA) regulated marker genes in *esr1-1* showed no increase in basal or SA induced expression suggesting repression of JA-regulated genes is not due to antagonistic SA-JA crosstalk. These results define new roles for KH-domain containing proteins with ESR1 unlinking JA-mediated growth and defense responses.

## Introduction

Plants are subject to constant changes in their environment and rapid molecular responses to these are necessary for plant survival. Upon detection of abiotic or biotic stress a series of induced signalling pathways are activated, mediated by key signalling hormones such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA), culminating in the expression of plant protectant and defense genes (reviewed by [1–8]). However, as multiple abiotic and biotic stresses can take place at the same time, a complex interplay of signalling pathways and responses can manifest resulting in opposing reactions. One mechanism to rapidly modify opposing stress-induced transcriptomes is to control the stability, degradation or turnover of specific transcripts at a post-transcriptional level through RNA-binding proteins.

RNA-binding proteins are mostly characterised by the presence of one or more RNA-binding domains. In addition to mRNA stability and decay, RNA-binding proteins are involved in diverse post-transcriptional processes including the maturation of mRNA through splicing, capping, polyadenylation and export from the nucleus [9, 10]. Along with plant specific processes such as flowering, the sessile nature of plants and a necessity to adapt quickly to changing environmental conditions may be why plants encode many RNA-binding, with over 200 RNA-binding proteins predicted in Arabidopsis [11]. Interestingly though, very few RNA-binding proteins have been functionally characterised in plants.

One group of genes expressed in response to biotic and abiotic stress are those belonging to the ubiquitous *GLUTATHIONE S-TRANSFERASE* (*GST*) enzyme family [12–15]. Plant *GST*s protect tissues against oxidative damage or from toxic products typically by catalyzing the conjugation of glutathione to a variety of electrophilic substrates of endogenous or exogenous origin, rendering the substrate less toxic (reviewed by [13, 15]). Expression of the Arabidopsis *GSTPH18* (*GSTF8*) gene is induced rapidly by diverse biotic and abiotic elicitors including pathogen attack, phytohormones, herbicides, heat and high-light stress, and as such has become a marker gene for early stress and defense responses [12, 16–23].

Using the *GSTF8* promoter to control the expression of a *Firefly Luciferase* reporter gene (*GSTF8:LUC*), we have been able to non-invasively monitor the plant's stress status, primarily within root tissues where *GSTF8* is predominantly expressed [17, 19, 21, 24]. To identify mechanisms controlling *GSTF8:LUC* activity we conducted a forward genetic screen using mutagenized populations of plants containing *GSTF8:LUC* [23]. One mutant isolated from this screen, designated *disrupted in stress responses* (*dsr1*), exhibited loss of SA inducible *GSTF8:LUC* activity and increased susceptibility to several fungal and bacterial pathogens [23]. The *dsr1* mutation was mapped to a single amino acid change in a subunit of the mitochondrial energy machinery (complex II subunit SDH1-1), causing a reduction in induced reactive oxygen species production (ROS) from mitochondria and identifying mitochondrial derived ROS as a critical component of plant defense [23].

To complement the *dsr1* study, we screened for mutants with enhanced *GSTF8:LUC* expression in the aim of identifying mutants with increased tolerance to biotic stress. We identified several alleles of a mutant called *enhanced stress response 1* (*esr1*) encoding a K homology (KH) domain containing RNA-binding protein (At5g53060). The *esr1* mutants confer constitutive *GSTF8:LUC* expression and increased resistance to the root-infecting fungal pathogen *Fusarium oxysporum*. Detailed analysis of the *esr1-1* allele also identified significant down-regulation of genes enriched for involvement in JA-mediated responses. Other mutants of *At5g53060* are reported to confer altered tolerance to abiotic stress [25–27] such as heat stress which we also established for *esr1-1*. While many Arabidopsis mutants conferring increased resistance to specific pathogens have been identified, these are commonly associated with a consequential decrease in tolerance to abiotic stress, or fitness costs such as poor growth or yield [2, 6, 28, 29].

By contrast, *esr1-1* displays increased *F. oxysporum* resistance, heat tolerance, and lacked observable defects in growth or development. These results define new roles for ESR1/At5g53060, functioning in biotic stress responses, JA-signalling, and unlinking growth restraint and resistance to stress.

## Materials and Methods

### Plant material and growth conditions

Unless otherwise specified, all experiments were conducted with the *Arabidopsis thaliana* Columbia-0 transgenic line (JC66/*GSTF8:LUC*) containing 791 bp of the *GSTF8* promoter fused to a luciferase reporter [17, 24]. Seeds were surface-sterilized, stratified at 4°C, and plated onto 100-mm square agar plates containing Murashige and Skoog (MS) salts as described previously [17]. Plates for luciferase assays were supplemented with 50  $\mu$ M luciferin (Biosynth AG). Plate and soil grown plants were incubated under a 16-h light/8-h dark cycle at 22°C. The T-DNA insertion mutant [30] used to generate *esr1-2* (SALK\_095666) was obtained from the Arabidopsis Biological Resource Centre (ABRC). For generation of plants expressing candidate *ESR1* genes the *At5g53060*, *At5g53150* and *At5g52860* CDS were amplified using primers listed in S1 Table. The resulting amplicons were cloned into BamHI/EcoRI digested binary vector pKEN [31] and confirmed by sequencing. 35S:*At5g53060* pKEN, 35S:*At5g53150* pKEN and 35S:*At5g52860* pKEN were mobilized into *Agrobacterium tumefaciens* AGL1 and transformed into *esr1-1* as per [31]. Transgenic plants were selected based on resistance to 10  $\mu$ g/mL glufosinate ammonium (Fluka). To generate *esr1-2*, SALK\_095666 and wild-type *GSTF8:LUC* lines were crossed and F<sub>3</sub> seedlings homozygous for the T-DNA and *GSTF8:LUC* selected.

### Bioluminescence and luciferase assays

Seedling bioluminescence was captured and quantified by imaging in an EG & G Berthold molecular light imager as previously described [17, 21]. Biochemical luciferase assays were performed as described by [24]. For 1 mM SA (Sigma) or temperature (45°C) treatments, 7-day old seedlings grown on square 100-mm Petri dishes were either covered with the liquid treatment at room temperature or incubated in temperature controlled cabinets for 40 min. After this time, excess liquid was discarded from relevant plates and the plates imaged such that, after acquiring the 0 hour bioluminescence image, 1 hour had elapsed.

### Mutant screen and mapping of *enhanced stress response 1*

Mutagenesis of wild-type *GSTF8:LUC* was described by [23]. For mapping, a genetic cross between *esr1-1* and Ler was generated and initial mapping conducted on 35 homozygous *esr1-1* F<sub>2</sub> plants (exhibiting constitutive *GSTF8:LUC* activity) with a set of 18 simple sequence-length polymorphism (SSLP) markers to map *esr1-1* to the bottom of chromosome 5, linked to marker *ciw9*. Additional mapping was performed by screening 1040 homozygous F<sub>2</sub> plants with markers listed in S1 Table.

### DNA isolation, Illumina sequencing, assembly and SNP annotation

DNA was extracted from backcrossed *esr1-1* using the CTAB method as described previously [32], followed by purifications using Agencourt AMPure XP beads (Beckman Coulter). Illumina Truseq DNA libraries were generated using manufactures recommendations and sequenced on an Illumina HiSeq1000 platform. Reads were trimmed, mapped against the TAIR10 release of the Arabidopsis genome [33] using bowtie2 v2.0.0b7 (parameters:-sensitive—end-to-end—met-stderr) [34] and SAMtools [35]. The aligned sequences were scanned for

SNPs relative to the TAIR10 reference using GATK (v2.1-6-g6a46042) [36]. The potential for SNP errors occurring around insertion-deletion regions was reduced using GATK Realigner-TargetCreator (parameters: `-windowSize 20 -minReadsAtLocus 2`) and IndelRealigner (parameters: `consensusDeterminationModel USE_SW -LODThresholdForCleaning 2 -maxconsensuses 100 -maxReadsForRealignment 100000 -maxReadsInMemory 300000`). Alignments were searched for SNPs using UnifiedGenotyper (parameters: `—stand_call_conf 50.0 -stand_emit_conf 10.0`). SNPs were considered as potentially contributing to the *esr1-1* phenotype if they resided within the *esr1-1* mapped loci. For *esr1-3* and *esr1-4*, pooled DNA from 50–60 homozygous F<sub>2</sub> plants from a Ler outcross were sequenced at 60–70x coverage by the Australian Genome Research Facility (AGRF) using an Illumina HiSeq Platform. Between 77.9 and 80.2 million paired-end reads (100 bp in length) per sample were mapped to the Arabidopsis TAIR10 genome reference sequence, SNPs called using the recommended SAMtools mpileup script and processed through the NGM tool <http://bar.utoronto.ca/ngm/> [37].

## Developmental and MeJA root elongation inhibition assays

Seeds of wild-type *GSTF8:LUC*, *esr1-1* and *esr1-2* were surface sterilized and plated onto MS media with germination rates measured as a percentage of total seeds plated ( $n = 60-70$ ). For root length and MeJA root elongation inhibition assays, seeds were sterilized as above and plated onto MS media in either the presence or absence of 25 or 50  $\mu\text{M}$  MeJA. Root length was measured on 7-day old seedlings using ImageJ [38]. Flowering time assays were conducted under long day conditions 16-h light/8-h dark cycle at 22°C ( $n = 10$ ).

## Pathogen assays

For *F. oxysporum* inoculations the isolate Fo5176 was used. Root-dip inoculations on 4-week-old plants with a  $1 \times 10^6$  cell/mL spore suspension were performed as described previously [39–41]. *A. brassicicola* assays were performed with isolate UQ4273 as described by [23]. A 5  $\mu\text{l}$  portion of a  $1 \times 10^6$  cell/mL spore suspension was applied to leaves of 3- to 4-week-old plants. Mock treatments with potato dextrose broth (PDB) were also conducted. Lesion size was measured with ImageJ [38]. For *R. solani* inoculations the strains AG2 or AG8 were used as previously described [23, 42]. 7-day old seedlings were sown into vermiculite and inoculated with 1 mL of  $1 \times 10^6$  cell/mL mycelium suspension.

## RNA isolation

For qRT-PCR and RNAseq experiments on untreated plants, tissue was collected from 4-, 7- or 14-day old seedlings grown vertically on MS agar plates. For gene expression under MeJA or SA treatment, 12-d-old seedlings germinated on MS plates were gently lifted into a mock medium (MS broth), 100  $\mu\text{M}$  MeJA medium (MS broth plus MeJA), or 1 mM SA (MS broth plus SA) such that the roots were submerged, and left for 6 or 24 h before harvesting. Three separate biological replicates were taken for all experiments consisting of whole seedlings pooled from 20–30 seedlings grown at the same time in the same environment, then frozen in liquid nitrogen, and stored at 80°C. RNA isolation was performed using the Qiagen RNeasy Plant Mini Kit (Qiagen). DNase treatment was performed after RNA isolation using TURBO DNase followed by treatment with the DNase Inactivation reagent (Ambion).

## qRT-PCR

Following RNA isolation and DNase treatment, complementary DNA synthesis was performed using SuperscriptIII reverse transcriptase (Invitrogen) with oligo(dT) (Invitrogen) and RNasin

(Promega) with 1 µg of input RNA. qRT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) on a CFX384 (Bio-Rad) system. Thermocycling and melt-curve conditions are described by [43]. Absolute gene expression levels relative to the previously validated [41, 44, 45] reference gene mix  $\beta$ -actin2,  $\beta$ -actin7, and  $\beta$ -actin8 (*At1g49240*, *At3g18780*, and *At5g09810*, respectively) were used for each complementary DNA sample using the equation: relative ratio gene of interest/actin =  $(E_{\text{gene}}^{-Ct_{\text{gene}}}) / (E_{\text{actin}}^{-Ct_{\text{actin}}})$  where Ct is the cycle threshold value. The  $\beta$ -actin mix contains reverse primers for each of the three  $\beta$ -actin genes and a universal forward primer. The mean expression range of the reference gene was found to be within  $\pm 1$  Ct across all samples. Several gene-specific primer sequences are previously published [45, 46] and are also listed in [S1 Table](#).

## RNA-seq library construction, Illumina sequencing and identification of differentially expressed genes

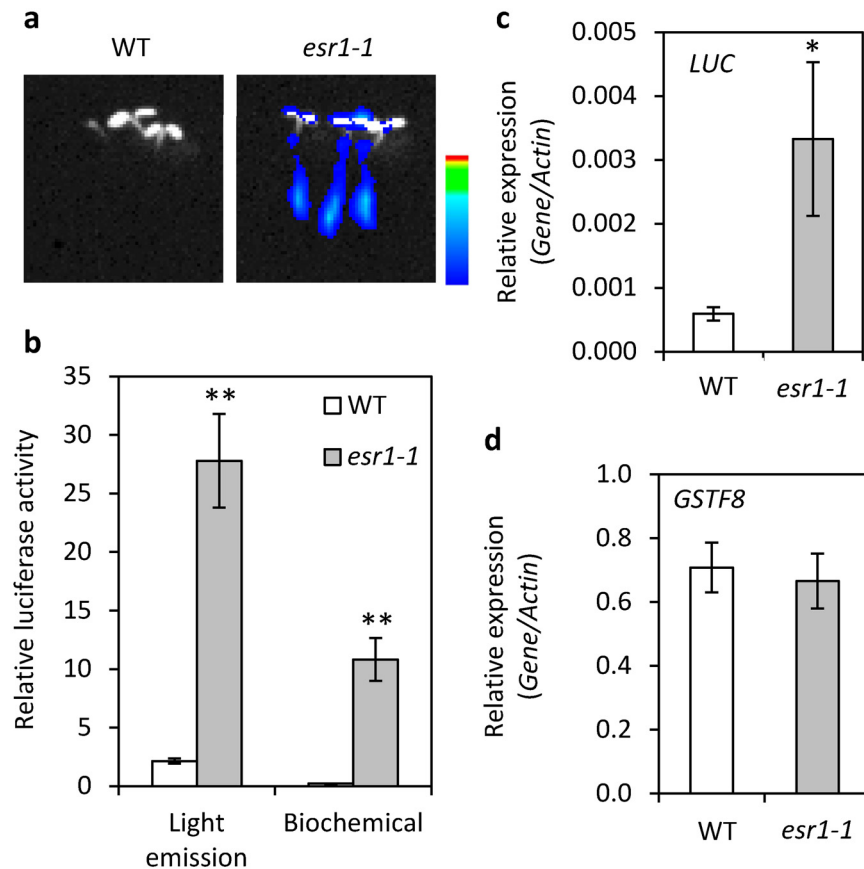
Following RNA isolation and DNase treatment of 14-day old wild-type or *esr1-1* samples, Illumina TruSeq libraries were generated from 1 µg of total RNA and sequenced on a HiSeq1000 platform (Illumina). RNA-seq paired-end reads were trimmed for low-quality base-calls and Illumina adapter sequences via Cutadapt (v1.1, parameters: `-quality-cutoff 30 -overlap 10 -times 3 -minimum-length 25`) [47]. Reads trimmed to less than 25 bp were discarded and remaining reads sorted into pairs and singleton reads. RNA-seq reads were mapped to the TAIR10 Arabidopsis genome reference [33] via Tophat (v2.0.9, parameters: `-b2-very-sensitive-r 50 -mate-std-dev 100 -i 20 -I 4000 -g 20 -report-secondary-alignments -report-discordant-pair-alignments -m 0 -min-coverage-intron 20 -coverage-search -microexon-search -library-type fr-unstranded`) [48]. RNAseq read alignments were supplied to Cuffdiff (Cufflinks v2.1.1) [49] to calculate normalised expression within the TAIR10 annotated genes as fragments per kilobase of transcript per million mapped fragments (FPKM) (parameters: `-frag-bias-correct -min-frags-per-fragment 4 -multi-read-correct`). Significantly differentially expressed transcripts between wild-type and *esr1-1* were detected from 3 biological replicates using Cuffdiff with default Benjamini-Hochberg correction for multiple-testing, based on a False Discovery Rate  $\leq 0.05$ . Functional annotations of genes and AGI symbols were sourced from TAIR10 datasets. RNA-seq reads have been deposited in the NCBI Sequence Read Archive under BioProject ID SRP056904.

## Results

### Identification of the constitutive *GSTF8:LUC* mutant *esr1-1*

To identify negative regulators of root stress responses we screened mutants from an ethyl methanesulfonate (EMS) mutagenised *GSTF8:LUC* population [23] for enhanced basal luciferase expression. Over 50 mutants with constitutive *GSTF8:LUC* expression were identified and termed *enhanced stress response* (*esr*) mutants. One of the mutants with the highest basal *GSTF8:LUC* expression (*esr1-1*) was further analysed and its phenotype confirmed in the  $M_3$  generation (Fig 1a and 1b). Quantitative real-time RT-PCR (qRT-PCR) was performed to confirm *LUCIFERASE* (*LUC*) gene expression and determine endogenous *GSTF8* expression. While *LUC* expression was up-regulated (5.6-fold greater than wild-type), *GSTF8* expression was unaltered (Fig 1c and 1d), suggesting the *esr1-1* mutation may only affect the *GSTF8:LUC* transgene and not endogenous *GSTF8* expression.

For cloning and heritability studies, we out-crossed *esr1-1* to the Landsberg *erecta* ecotype (Ler). All  $F_1$  plants showed the wild-type phenotype, and  $F_2$  plants displayed a  $\sim 3:1$  segregation (59:21,  $\chi^2$  test  $p = 0.8$ ) suggesting the *esr1-1* phenotype is due to a recessive mutation in a single nuclear gene.

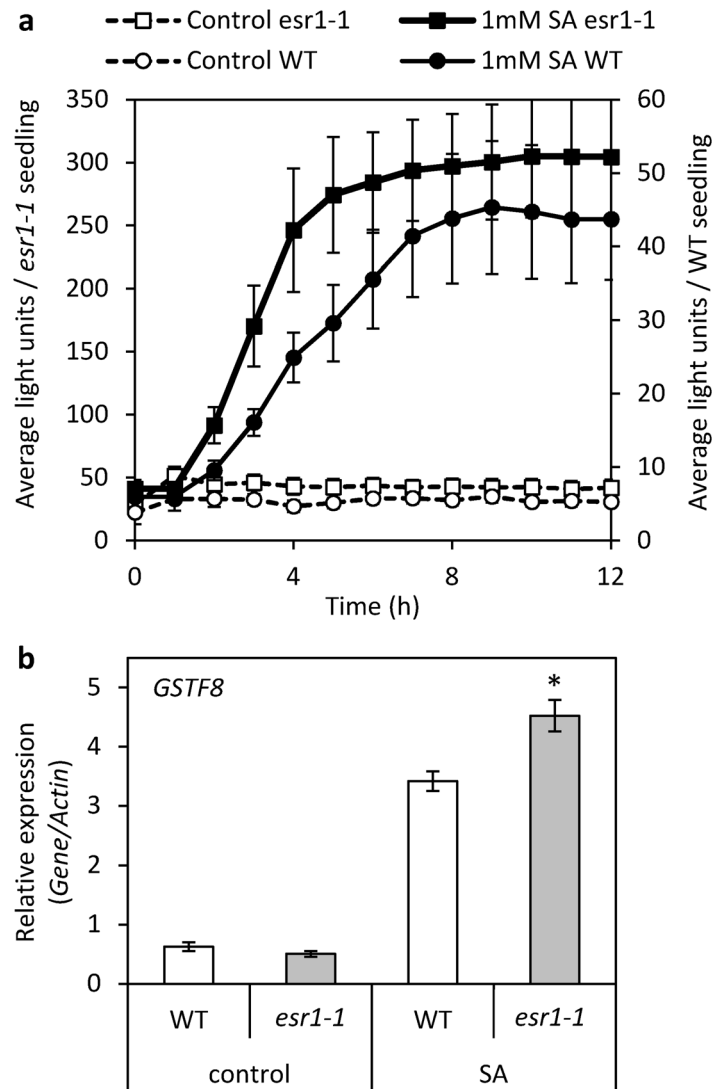


**Fig 1. *esr1-1* causes hyper-expression of basal *GSTF8:LUC* activity.** (a) *GSTF8:LUC* expression in 4 day old wild-type (WT) and *esr1-1* seedlings. Shown is bioluminescence (pseudocolored blue) superimposed onto a fluorescence (white) image. Intensity of bioluminescence ranges from blue to red as depicted in the intensity ruler. (b) Quantification of bioluminescence via *in vivo* light emission (relative light units/seedling; values are averages  $\pm$  SE (n = 30) from 4 day old seedlings) and *in vitro* biochemical assays (units/20sec/mg protein; values are averages  $\pm$  SE (n = 30) from 9 day old seedlings). (c-d) *Luciferase (LUC)* and *GSTF8* expression in 4 day old seedlings (values are averages  $\pm$  SE of 4 biological replicates consisting of pools of 20 seedlings). Gene expression levels are relative to the internal control  $\beta$ -*actin* genes. Asterisks indicate values that are significantly different (\*\* $P < 0.01$ , \* $P < 0.05$  Student's *t*-test) from WT.

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### *GSTF8:LUC* activity and endogenous *GSTF8* expression is up-regulated in *esr1-1* following SA treatment

To further characterise *esr1-1*, we monitored *GSTF8:LUC* expression following SA treatment, known to rapidly induce *GSTF8* promoter activity in wild-type plants [17, 24]. *GSTF8:LUC* activity increased more rapidly in *esr1-1* following SA treatment where it plateaued at 6–7 hours post treatment compared to wild-type seedlings where this occurred at 8–9 hours (Fig 2a). Expression of the endogenous *GSTF8* gene in *esr1-1* under SA-inducing conditions was also significantly higher in *esr1-1* compared to wild-type (Fig 2b). Combined with the lack of increased basal *GSTF8* expression in *esr1-1* (Fig 1d), these results suggest regulation of basal but not stress inducible *GSTF8 promoter:LUC* activity differs from the context of the endogenous *GSTF8* gene, possibly due to regulatory components beyond the promoter fragment used in this study.

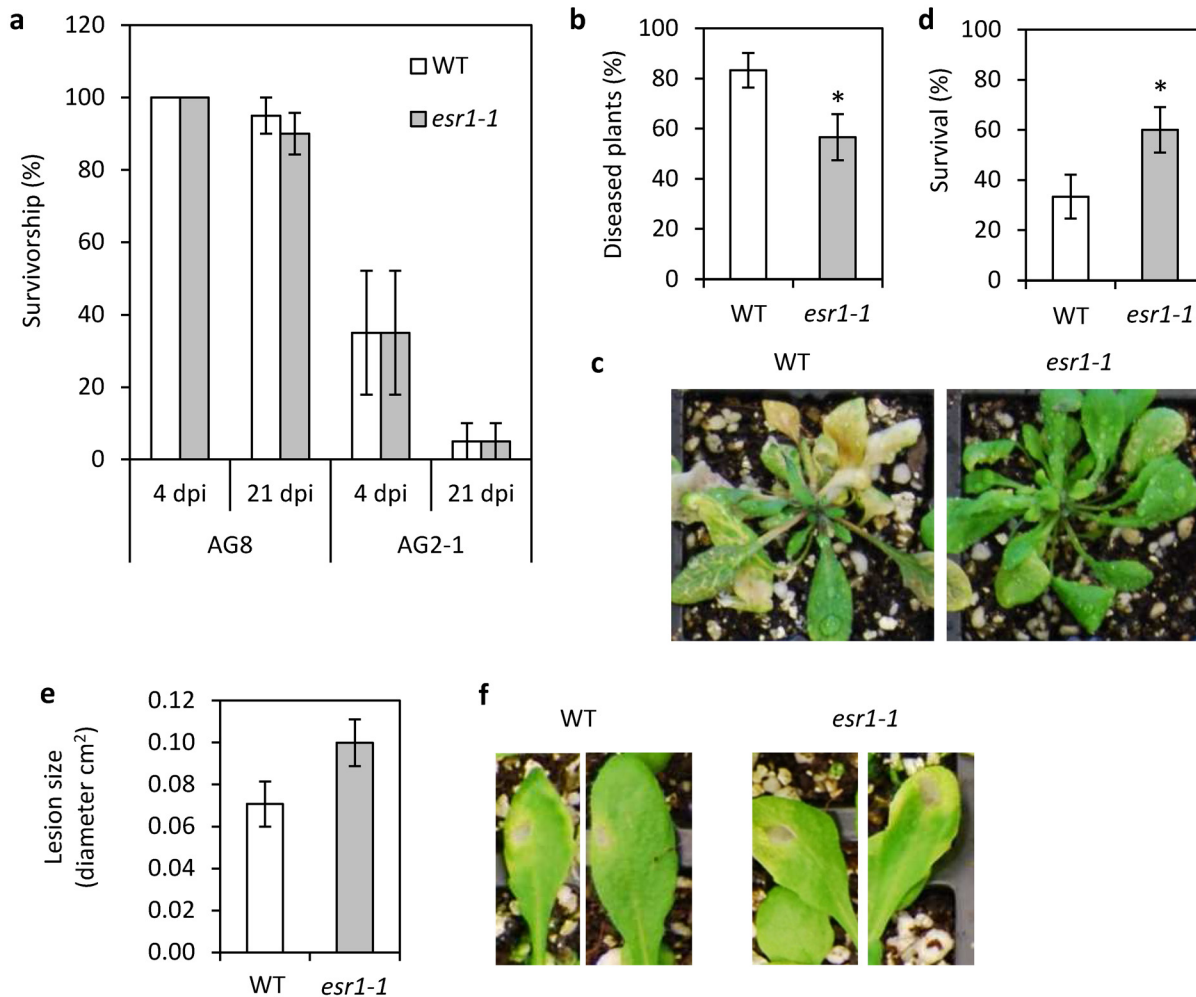


**Fig 2. *GSTF8:LUC* activity and endogenous *GSTF8* expression is up-regulated in *esr1-1* following SA treatment.** (a) Average *GSTF8:LUC* expression per wild-type (WT) and *esr1-1* seedling per hour after treatment with 1mM salicylic acid (SA) or a control treatment. Values are averages  $\pm$  SE (n = 5) from 7 day old seedlings with *esr1-1* and WT values plotted on the left and right axes respectively. Similar results were obtained in independent experiments. (b) *GSTF8* expression in 12 day old seedlings 6 hours post control or SA treatment (values are averages  $\pm$  SE of 3 biological replicates consisting of pools of 20–30 seedlings). Gene expression levels are relative to the internal control  $\beta$ -actin genes. Asterisks indicate values that are significantly different (\* $P < 0.05$  Student's *t*-test) from WT.

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## ESR1 is a negative regulator of resistance to the fungal pathogen *Fusarium oxysporum*

We previously identified a mutant from the same screen as *esr1-1* but with loss of SA inducible *GSTF8:LUC* activity. This mutant termed *disrupted in stress responses 1 (dsr1)* exhibits increased susceptibility to several pathogens [23]. As *esr1-1* exhibits increased root localised *GSTF8:LUC* expression (Figs 1 and 2), we hypothesized *esr1-1* may confer increased resistance to root pathogens. To test this, we first inoculated wild-type and *esr1-1* plants with the root-infecting fungal pathogens *Rhizoctonia solani* and *Fusarium oxysporum* [19, 50]. While no significant difference



**Fig 3. *esr1-1* exhibits increased resistance to *Fusarium oxysporum*.** (a) Percentage survivorship of wild-type (WT) and *esr1-1* seedlings at 4 and 21 days post inoculation (dpi) with *Rhizoctonia solani* isolates AG8 and AG2-1. Values are averages  $\pm$  SE of 4 biological replicates consisting of pools of 5 seedlings. (b-d) Disease phenotypes of *F. oxysporum* inoculated plants with (b) percentage and (c) representative images of diseased plants 10 days post inoculation. (d) Survival at 21 days post inoculation. Values are averages  $\pm$  SE ( $n = 30$ ). (e-f) *A. brassicicola* induced lesions on (e) WT and *esr1-1* leaves 3 days post inoculation with (f) representative images of leaves. Values are averages  $\pm$  SE of 6 biological replicates consisting of lesion diameters measured from 4 inoculated leaves per plant. Asterisks indicate values that are significantly different ( $*P < 0.05$  Student's *t*-test) from WT. Similar results were obtained in independent experiments.

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in disease symptom development or survivorship was observed between wild-type or *esr1-1* plants inoculated with *R. solani* (strains AG2 or AG8) (Fig 3a), *esr1-1* did exhibit increased resistance to *F. oxysporum* (Fig 3b–3d). This was observed through both a delay in disease symptom development and increased survival. While jasmonate (JA)-mediated defences are required for resistance to most fungal necrotrophic pathogens (e.g. *Botrytis cinerea*, *Alternaria brassicicola*, [51]), JA-signalling confers susceptibility to *F. oxysporum* with mutants compromised in JA-dependant responses exhibiting resistance to this pathogen [41, 45, 52].

The increased resistance to *F. oxysporum* prompted us to determine if *esr1-1* conferred increased susceptibility to *A. brassicicola*. Larger *A. brassicicola* induced lesions were observed on *esr1-1* leaves compared to wild-type (Fig 3e and 3f), however not a statistically significant level. This phenotype was observed over several independent experiments suggesting ESR1 contributes a small affect to inhibition of *A. brassicicola* lesion development. As could be hypothesized



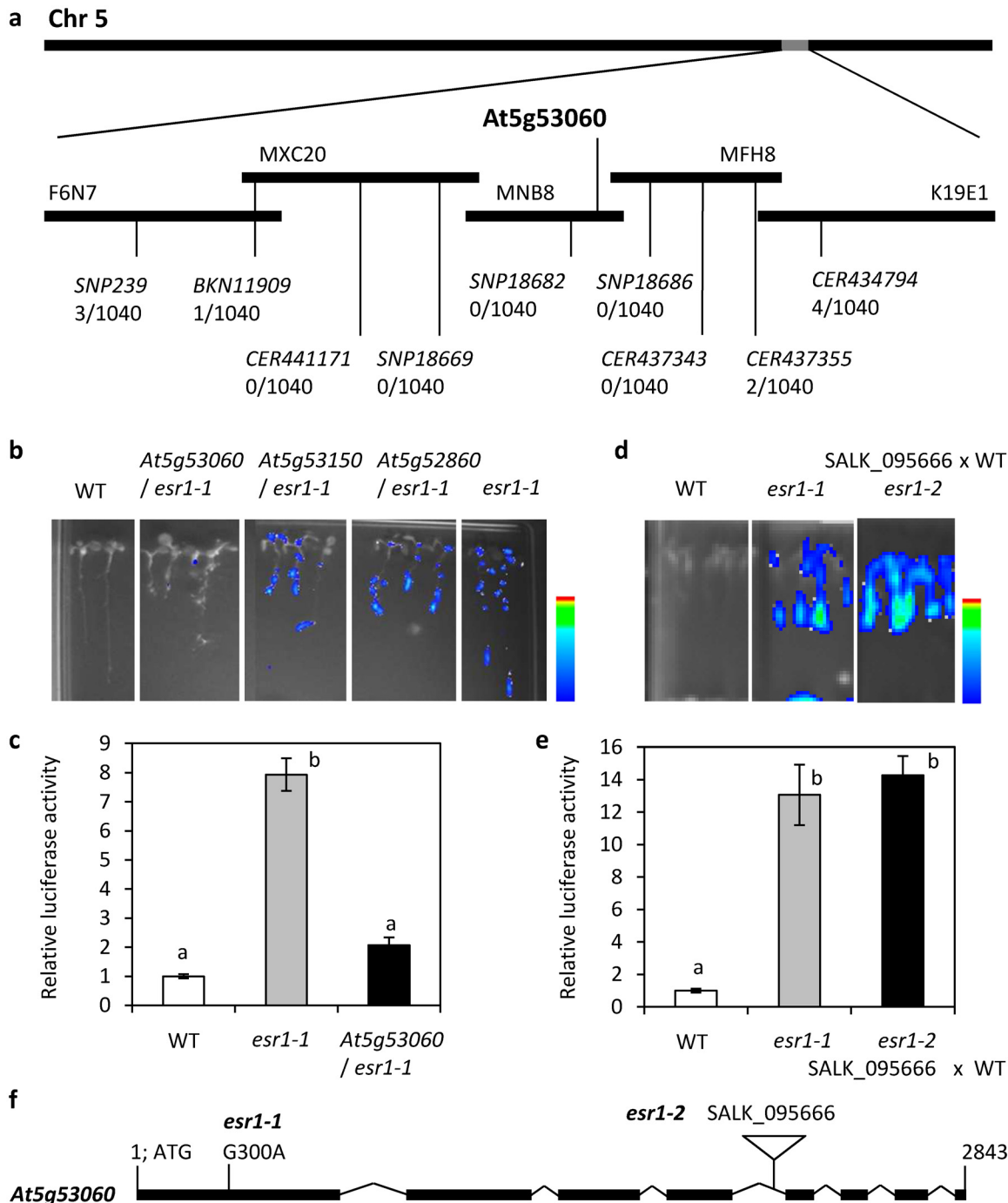
from the *F. oxysporum* results, increased *A. brassicicola* induced lesions may be due to reduced JA-responses in *esr1-1*.

### *ESR1* encodes a K homology (KH) domain RNA-binding protein

To determine the causal *esr1-1* mutation, map based cloning was initiated using F<sub>2</sub> seeds of the *esr1-1* and Ler cross. Genetic mapping was conducted on 1040 homozygous F<sub>2</sub> plants. The mutation was narrowed down to a region on Chromosome 5 spanning ~200 Kb across three Bacterial Artificial Chromosomes (BACs); MXC20, MNB8 and MFH8 (Fig 4a). Whole genome sequencing of the *GSTF8:LUC* wild-type parent and *esr1-1* and alignment to the TAIR10 genome identified five single nucleotide polymorphisms (SNPs) within the mapped loci with three causing non-synonymous mutations in gene coding regions. Molecular complementation assays were conducted by individually introducing the three candidate genes under the control of the 35S promoter into the *esr1-1* background. The 35S:*At5g53060* construct eliminated the *esr1-1* constitutive *GSTF8:LUC* expression and restored the wild-type phenotype, suggesting a G300A nucleotide change in *At5g53060* and resulting stop codon substitution of W100\* causes the *esr1-1* phenotype (Fig 4b, 4c and 4f). *At5g53060* encodes a heterogenous nuclear riboprotein (hnRNP) K homology (KH) domain containing RNA-binding protein. The protein contains five KH-domains and the *esr1-1* mutation disrupts the first of these domains. KH-domains are found in many RNA-binding proteins and are associated with transcriptional and post-transcriptional processes where they can bind RNA or single stranded DNA [11, 53, 54]. In all subsequent experiments the *esr1-1* line used is a line backcrossed twice to the wild-type parent (to remove other EMS induced mutations) and has the same *GSTF8:LUC* phenotype as the M<sub>3</sub> *esr1-1* line (data not shown).

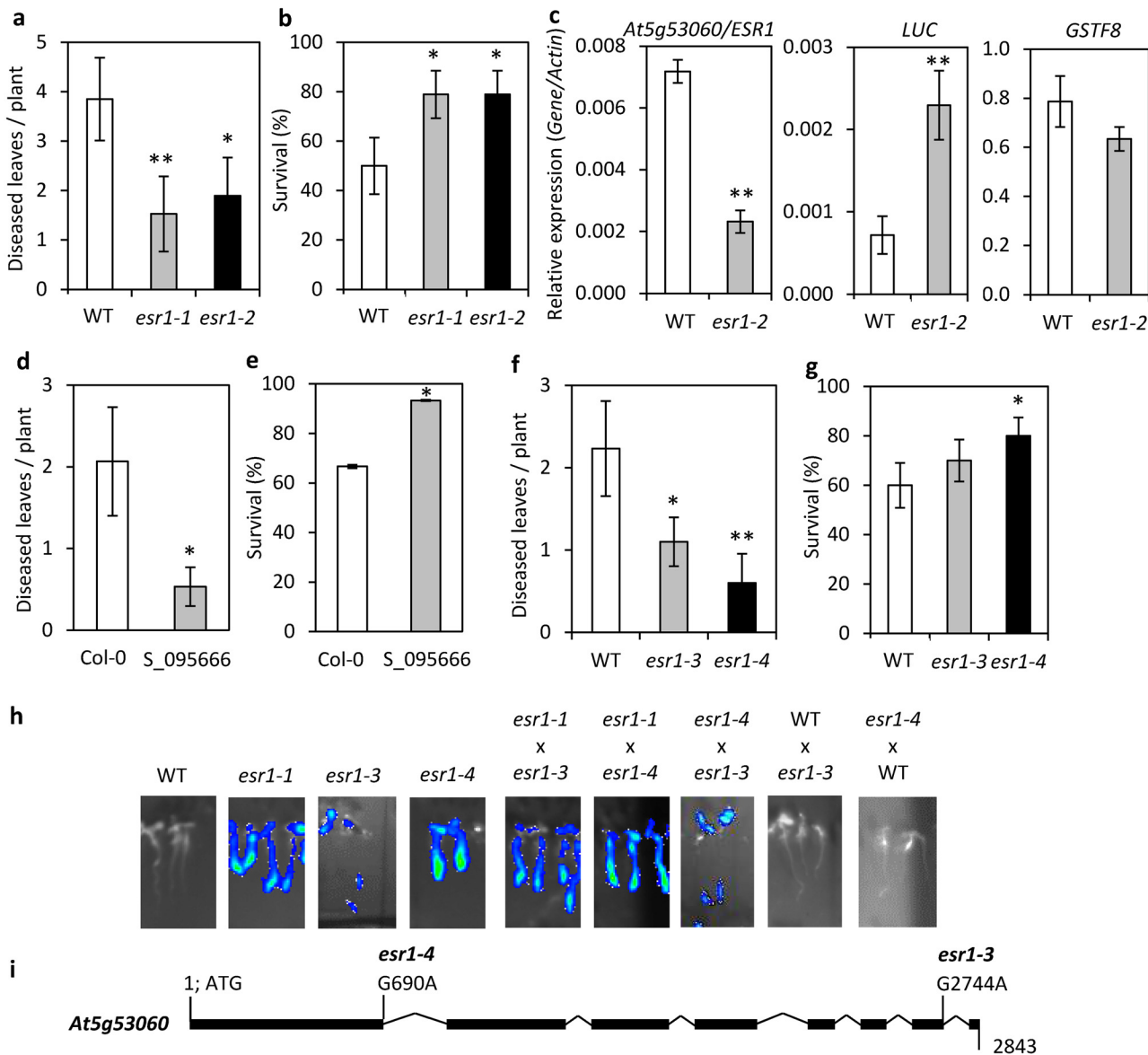
To confirm a mutation in *At5g53060* is responsible for the *esr1-1* increased *GSTF8:LUC* and *Fusarium* resistance phenotypes, a T-DNA insertion line in *At5g53060* (SALK\_095666) was crossed with wild-type *GSTF8:LUC*. We generated F<sub>3</sub> seedlings homozygous for the T-DNA and *GSTF8:LUC* (subsequently named *esr1-2*) which displayed both the constitutive *esr1-1* luciferase phenotype and increased resistance to *F. oxysporum*, further supporting that the *esr1-1* mutant phenotypes result from disruption of *At5g53060* (Figs 4d–4f, 5a and 5b). As with *esr1-1*, *esr1-2* plants exhibited increased basal expression of *LUCIFERASE* but not *GSTF8* (Fig 5c). To rule out the possibility that increased transgene activity in the *esr1* mutants contributes to *Fusarium* resistance, we inoculated SALK\_095666 in the absence of the *GSTF8:LUC* transgene, and included control Col-0 plants. As with both *esr1-1* and *esr1-2* mutants, the SALK\_095666 line also showed significantly increased resistance to *Fusarium* (Fig 5d and 5e). These results therefore confer a new role for *At5g53060*/ESR1, in mediating responses to biotic stress.

Of the other 50 constitutive *GSTF8:LUC* mutants isolated from our initial screen, we identified two further recessive alleles of *esr1* using the process of Next Generation Mapping (NGM) [37]. Using the NGM tool, SNPs were called against the TAIR10 genome reference and identified a region on chromosome 5 low in heterozygosity, incidentally mapping to the *ESR1/At5g53060* loci (S1 Fig). We found both mutants conferred increased resistance to *F. oxysporum* and through genetic complementation confirmed both mutants were indeed alleles of *esr1*, subsequently designating them as *esr1-3* and *esr1-4* (Fig 5f–5h). *esr1-3* confers a G2744A change at the second last exon/intron boundary (TGAGCAgtaagt>TGAGCAaataagt), while *esr1-4* confers a G690A change at the last codon of the first exon causing a synonymous change (CAG>CAA, Q>Q) (Fig 5i). Processing of these mutated genes through the splice site prediction software NetGene2 (version 2.4; [55]) revealed that both mutants would encode misspliced transcripts with *esr1-3* losing the last donor splice site and *esr1-4* losing the first donor splice site.



**Fig 4. Molecular cloning of *esr1-1*.** (a) Fine mapping of *esr1-1* with recombination events from 1040 plants analysed for each marker shown. (b-c) Wild-type (WT) and *esr1-1* genomes were sequenced and inspected for SNP differences within the mapped loci to identify 3 candidate genes. Molecular complementation of the *esr1-1* mutation by the wild-type *At5g53060* gene with (b) images and (c) luciferase quantification shown ( $P < 0.05$ , all pairs Student's t-test). (d-e) Genetic complementation between *esr1-1* and a *At5g53060* T-DNA knockout (SALK\_095666) with (d) images and (e) luciferase quantification shown ( $P < 0.05$ , all pairs Student's t-test). The *esr1-2* mutant is an  $F_3$  line from a cross between WT plants and the T-DNA insertion line SALK\_095666 and is homozygous for the T-DNA insertion and *GSTF8:LUC* transgene. (f) Structure of the *At5g53060* gene with *esr1-1* mutation and T-DNA knockout locations indicated. Filled boxes indicate exons, joining lines indicate introns. Positions are relative to the start codon.

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**Fig 5. Identification and screening of other *esr1* alleles/insertion lines conferring increased resistance to *Fusarium oxysporum*.** (a-b) Disease phenotypes of *F. oxysporum* inoculated wild-type (WT) *GSTF8:LUC* and *esr1-2* plants. Values are averages  $\pm$  SE ( $n > 15$ ). (c) *At5g53060/ESR1*, *LUCIFERASE (LUC)* and *GSTF8* expression in 12 day old WT and *esr1-2* seedlings (values are averages  $\pm$  SE of 3 biological replicates consisting of pools of 20 seedlings). Gene expression levels are relative to the internal control  $\beta$ -actin genes. (d-g) Disease phenotypes of *F. oxysporum* inoculated (d-e) Col-0 and SALK\_09566, and (f-g) WT *GSTF8:LUC* and *esr1-3* and *esr1-4* plants. Values are averages  $\pm$  SE ( $n > 15$ ). (h) *esr1-1*, *esr1-3* and *esr1-4* mutants were crossed and F<sub>1</sub> progeny screened for complementation of the *GSTF8:LUC* constitutive expression phenotype. Crosses to wild-type (WT) *GSTF8:LUC* were included as controls. (i) Next Generation Mapping identified *esr1-3* and *esr1-4* mutations at splice site junctions in *At5g53060/ESR1*. For *Fusarium* disease assays, diseased leaves was measured at 14 days post inoculation and survival at 21 days post inoculation. Asterisks indicate values that are significantly different (\*\* $P < 0.01$ , \* $P < 0.05$  Student's *t*-test) from WT or Col-0.

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### *esr1* mutants confer increased tolerance to heat stress

Three other mutant alleles of *At5g53060* have been independently identified through abiotic stress screens using salt, desiccation or cold-inducible promoters and confer increased or reduced tolerance to specific abiotic stresses. These are *Regulator of CBF gene expression 3 (rcf3-1)*, [26], *Shiny1 (shi1)*, [27], and *High Osmotic Stress Gene Expression 5 (hos5-1)*, [56]. To

determine if *esr1-1* exhibited altered tolerance to abiotic stress we conducted heat tolerance assays described by [26] and found *esr1-1* also conferred increased tolerance to heat stress (S2 Fig). This was also confirmed for the insertional knockout mutant *esr1-2* (S2 Fig). To further characterise the role of At5g53060/ESR1 in temperature stress, we monitored *GSTF8:LUC* activity in wild-type and *esr1-1* seedlings over a 12 h time-course following heat stress. As with results following SA treatment (Fig 2), *GSTF8:LUC* activity increased more rapidly in *esr1-1* compared to wild-type seedlings (S2 Fig) suggesting ESR1 contributes to the repression of SA and heat induced stress responses.

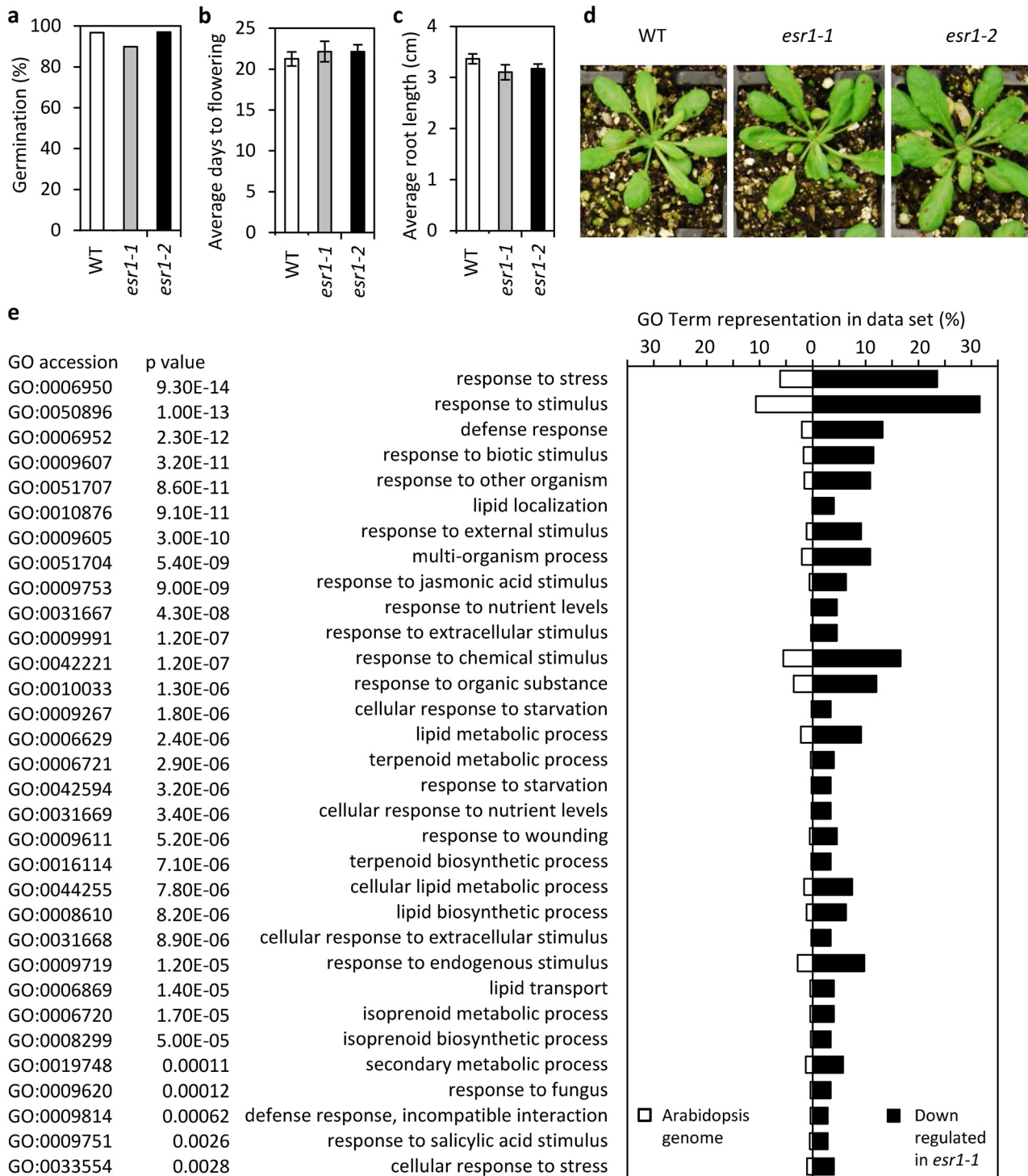
## Whole transcriptome sequencing of *esr1-1* reveals altered expression of genes involved in biotic and abiotic stress responses

To uncover the possible direct or indirect targets of At5g53060/ESR1, we performed whole-transcriptome sequencing (RNA-seq) on three biological replicates of *esr1-1* and wild-type seedlings using the Illumina HiSeq platform. We used un-treated seedlings as *GSTF8:LUC* is constitutively up-regulated in *esr1-1* under normal growing conditions (Fig 1a). Between 18.5 and 21.4 million paired-end reads (100 bp in length) per sample were mapped to the Arabidopsis TAIR10 exome reference sequence.

Using the Cuffdiff tool within Cufflinks [49] we identified 1176 significantly differentially expressed genes between wild-type and *esr1-1* (Benjamini-Hochberg correction for multiple-testing based on a False Discovery Rate  $\leq 0.05$ ). Based on significant FPKMs (Fragments Per Kilobase of exon model per Million mapped fragments) fold changes, more transcripts were down-regulated in *esr1-1* (873) compared to up-regulated (303) (S2 and S3 Tables). To gain insight into the functions of these genes we performed Gene Ontology (GO) term enrichment analysis using agriGO v1.2 [57] with the default False Discovery Rate ( $p \leq 0.05$ ) determined p-value significance. Among the most significantly enriched biological processes from genes down-regulated in *esr1-1* were those involved in response to stress, biotic and abiotic stimuli, defense responses, wounding responses, and response to other organisms (bacteria and fungi) (S4 Table). Among the most significantly enriched biological processes from genes up-regulated in *esr1-1* were those involved in response to light, abiotic and hormone stimulus, cell death, signaling pathways and developmental processes (S5 Table). While *esr1-1* displays these significant changes in stress and defense responsive gene expression, interestingly neither *esr1-1* or *esr1-2* show obvious deleterious growth or developmental defects (Fig 6a–6d) often exhibited by Arabidopsis mutants with similar expression profiles [2, 58].

To consolidate a smaller list of differentially expressed genes for follow up qRT-PCR studies we sorted the gene lists for those with significantly altered expression greater than 2-fold from wild-type levels. This identified 48 genes up-regulated in *esr1-1* and 174 genes down-regulated (Tables 1 and 2). Although analysis of the 48 up-regulated genes did not reveal any specific GO term enrichment for biological processes, there was molecular function enrichment for nucleoside and nucleotide binding. The gene list included C-Terminal Domain Phosphatase-Like1 (CPL1) which can physically interact with At5g53060 [27, 56, 59] and was one of the most significantly up-regulated genes in *esr1-1*. Other genes with strong up-regulation were *At3g54160* an uncharacterised F-box/RNI-like protein, *SYNAPTOTAGMIN 2* (*SYTB*) a calcium-dependent lipid-binding protein involved in protein secretion, and *SUGAR TRANSPORTER 11* (*STP11*). Examination of these three genes and *CPL1* using qRT-PCR within a developmental time-course comparing wild-type versus *esr1-1* seedlings, including the 14-day old seedlings that were sampled for the RNAseq analysis, revealed their constitutive up-regulation at all time-points (S3 Fig).

We next examined the list of 174 genes that were significantly down-regulated in *esr1-1* >2-fold over wild-type (Table 2) for GO term enrichment. Amongst the most significantly



**Fig 6. Significant enrichment of stress and defense associated biological process Gene Ontology (GO) terms in *esr1-1* down-regulated genes is not associated with developmental impairment.** (a-d) Neither *esr1-1* nor *esr1-2* differ from wild-type in (a) germination, (b) flowering time, (c) root or (d) leaf development. (e) Genes significantly down-regulated  $\geq 2$ -fold in *esr1-1* (compared to wild-type) were analyzed for enrichment of GO terms associated with biological processes. Shown are GO term representations in the *esr1-1* dataset compared to representation in the Arabidopsis genome. GO terms are ordered by p values adjusted by the False Discovery Rate.

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**Table 1. *esr1-1* ≥2-fold up-regulated genes.**

| AGI locus | symbol   | fold change ( <i>esr1-1</i> /WT) | FDR-adjusted p-value | AGI description   |
|-----------|----------|----------------------------------|----------------------|---|
| AT4G21670 | CPL1     | 2.43                             | 0                    | C-terminal domain phosphatase-like 1  |
| AT1G17990 |          | 2.65                             | 0                    | FMN-linked oxidoreductases superfamily protein                                      |
| AT5G25280 |          | <b>3.15</b>                      | 0                    | serine-rich protein-related   |
| AT5G25130 | CYP71B12 | 2.16                             | 3.6E-15              | cytochrome P450, family 71, subfamily B, polypeptide 12                             |
| AT2G01860 | EMB975   | 2.48                             | 3.3E-11              | Tetratricopeptide repeat (TPR)-like superfamily protein, EMBRYO DEFECTIVE 975       |
| AT1G03990 |          | <b>4.15</b>                      | 1.9E-09              | Long-chain fatty alcohol dehydrogenase family protein                               |
| AT5G66520 |          | 2.39                             | 1.2E-08              | Tetratricopeptide repeat (TPR)-like superfamily protein                             |
| AT1G19340 |          | 2.75                             | 2.1E-06              | Methyltransferase MT-A70 family protein   |
| AT3G54160 |          | <b>14.45</b>                     | 2.4E-05              | RNI-like superfamily protein  |
| AT5G44120 | CRA1     | 2.07                             | 8.3E-05              | RmlC-like cupins superfamily protein, CRUCIFERINA                                   |
| AT2G36790 | UGT73C6  | 2.07                             | 1.2E-04              | UDP-glucosyl transferase 73C6   |
| AT1G61400 |          | 2.78                             | 1.5E-04              | S-locus lectin protein kinase family protein  |
| AT5G11470 |          | 2.35                             | 2.5E-04              | bromo-adjacent homology (BAH) domain-containing protein                             |
| AT2G42730 |          | 2.34                             | 2.5E-04              | F-box family protein  |
| AT5G44980 |          | <b>3.55</b>                      | 3.2E-04              | F-box/RNI-like/FBD-like domains-containing protein                                  |
| AT1G50790 |          | 2.55                             | 3.8E-04              | Plant mobile domain protein family  |
| AT1G61500 |          | 2.09                             | 7.6E-04              | S-locus lectin protein kinase family protein  |
| AT1G77960 |          | <b>3.26</b>                      | 1.3E-03              | Unknown   |
| AT2G30505 |          | 2.91                             | 1.3E-03              | Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family           |
| AT3G57580 |          | 2.11                             | 2.0E-03              | F-box and associated interaction domains-containing protein                         |
| AT3G53680 |          | 2.02                             | 2.9E-03              | Acyl-CoA N-acyltransferase with RING/FYVE/PHD-type zinc finger domain               |
| AT1G52990 |          | <b>7.45</b>                      | 2.9E-03              | thioredoxin family protein  |
| AT1G75110 | RRA2     | 2.11                             | 4.2E-03              | Nucleotide-diphospho-sugar transferase family protein, REDUCED RESIDUAL ARABINOSE 2 |
| AT3G26550 |          | 2.08                             | 5.4E-03              | Cysteine/Histidine-rich C1 domain family protein                                    |
| AT4G03440 |          | 2.03                             | 5.4E-03              | Ankyrin repeat family protein   |
| AT4G28520 | CRU3     | 2.01                             | 5.6E-03              | cruciferin 3  |
| AT3G44713 |          | 3.25                             | 5.7E-03              | Unknown   |
| AT1G13609 |          | 2.16                             | 6.3E-03              | Defensin-like (DEFL) family protein   |
| AT2G07732 |          | 2.05                             | 6.5E-03              | Ribulose biphosphate carboxylase large chain, catalytic domain                      |
| AT1G12700 |          | 2.07                             | 7.0E-03              | ATP binding;nucleic acid binding;helicases  |
| AT3G21370 | BGLU19   | <b>3.89</b>                      | 7.6E-03              | beta glucosidase 19   |
| AT5G37750 |          | <b>3.70</b>                      | 8.8E-03              | Chaperone DnaJ-domain superfamily protein   |
| AT1G51520 |          | 2.06                             | 9.4E-03              | RNA-binding (RRM/RBD/RNP motifs) family protein                                     |
| AT5G37400 |          | 2.15                             | 9.4E-03              | Family of unknown function (DUF577)   |
| AT3G18970 | MEF20    | 2.18                             | 1.0E-02              | mitochondrial editing factor 20   |
| AT1G20080 | SYTB     | <b>7.60</b>                      | 1.1E-02              | Calcium-dependent lipid-binding (CaLB domain) family protein                        |
| AT5G23270 | STP11    | <b>6.74</b>                      | 1.2E-02              | sugar transporter 11  |
| AT2G19910 |          | 2.15                             | 1.3E-02              | RNA-dependent RNA polymerase family protein   |
| AT3G47090 |          | 2.09                             | 1.5E-02              | Leucine-rich repeat protein kinase family protein                                   |
| AT5G38040 |          | 2.15                             | 1.8E-02              | UDP-Glycosyltransferase superfamily protein   |
| AT5G23600 |          | 2.21                             | 2.0E-02              | RNA 2'-phosphotransferase, Tpt1 / KptA family                                       |
| AT3G46370 |          | 2.13                             | 2.3E-02              | Leucine-rich repeat protein kinase family protein                                   |
| AT4G16940 |          | 2.12                             | 2.6E-02              | Disease resistance protein (TIR-NBS-LRR class) family                               |
| AT5G43840 | HSFA6A   | 2.74                             | 3.2E-02              | heat shock transcription factor A6A   |
| AT5G66960 |          | 2.02                             | 3.5E-02              | Prolyl oligopeptidase family protein  |

(Continued)

Table 1. (Continued)

| AGI locus | symbol | fold change ( <i>esr1-1</i> /WT) | FDR-adjusted p-value | AGI description   |
|-----------|--------|----------------------------------|----------------------|---|
| AT4G38010 |        | 2.59                             | 4.0E-02              | Pentatricopeptide repeat (PPR-like) superfamily protein |
| AT1G58320 |        | 2.14                             | 4.0E-02              | PLAC8 family protein                                    |
| AT4G10600 |        | 2.66                             | 4.1E-02              | RING/FYVE/PHD zinc finger superfamily protein           |

Fold change based on FPKMs from 3 biological replicates. Significance based on Benjamini-Hochberg correction for multiple-testing,  $P \leq 0.05$  adjusted by the False Discovery Rate. Values in bold are  $\geq 3$ -fold changes.

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Table 2. *esr1-1*  $\geq 2$ -fold down-regulated genes.

| AGI locus | symbol   | fold change ( <i>esr1-1</i> /WT) | FDR-adjusted p-value | AGI description  |
|-----------|----------|----------------------------------|----------------------|--|
| AT3G49620 | DIN11    | <b>15.9</b>                      | 0                    | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase protein, DARK INDUCIBLE 11                   |
| AT2G39030 | NATA1    | <b>8.6</b>                       | 0                    | Acyl-CoA N-acyltransferases (NAT) superfamily protein  |
| AT5G42600 | MRN1     | <b>6.1</b>                       | 0                    | marneral synthase  |
| AT3G12740 | ALIS1    | <b>4.8</b>                       | 0                    | ALA-interacting subunit 1  |
| AT4G15210 | BAM5     | <b>4.5</b>                       | 0                    | beta-amylase 5   |
| AT2G39330 | JAL23    | <b>3.8</b>                       | 0                    | jacalin-related lectin 23  |
| AT3G57260 | BGL2     | <b>3.4</b>                       | 0                    | beta-1,3-glucanase 2   |
| AT3G25830 | TPS-CIN  | <b>3.3</b>                       | 0                    | terpene synthase-like sequence-1,8-cineole   |
| AT1G33960 | AIG1     | <b>3.3</b>                       | 0                    | P-loop containing nucleoside triphosphate hydrolases superfamily protein, AVRRPT2-INDUCED GENE 1 |
| AT2G24850 | TAT3     | <b>3.1</b>                       | 0                    | tyrosine aminotransferase 3  |
| AT1G45201 | TLL1     | 2.9                              | 0                    | triacylglycerol lipase-like 1  |
| AT5G20150 | SPX1     | 2.9                              | 0                    | SPX domain gene 1  |
| AT2G43510 | TI1      | 2.6                              | 0                    | trypsin inhibitor protein 1  |
| AT2G39310 | JAL22    | 2.5                              | 0                    | jacalin-related lectin 22  |
| AT3G04720 | PR4      | 2.4                              | 0                    | pathogenesis-related 4   |
| AT1G19670 | CLH1     | 2.3                              | 0                    | chlorophyllase 1   |
| AT5G24770 | VSP2     | 2.3                              | 0                    | vegetative storage protein 2   |
| AT5G48850 | ATSDI1   | 2.1                              | 0                    | Tetratricopeptide repeat (TPR)-like superfamily protein, SULPHUR DEFICIENCY-INDUCED 1            |
| AT3G45140 | LOX2     | 2.1                              | 0                    | lipoxygenase 2   |
| AT1G73260 | KTI1     | 2.0                              | 0                    | kunitz trypsin inhibitor 1   |
| AT4G22517 |          | <b>3.5</b>                       | 3.6E-15              | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein        |
| AT2G43530 |          | 2.8                              | 3.6E-15              | Scorpion toxin-like knottin superfamily protein  |
| AT5G24780 | VSP1     | 2.5                              | 7.2E-15              | vegetative storage protein 1   |
| AT3G21500 | DXPS1    | <b>6.7</b>                       | 1.8E-14              | 1-deoxy-D-xylulose 5-phosphate synthase 1  |
| AT3G49580 | LSU1     | 2.5                              | 3.4E-14              | response to low sulfur 1   |
| AT5G42590 | CYP71A16 | 2.4                              | 5.7E-14              | cytochrome P450, family 71, subfamily A, polypeptide 16  |
| AT4G12470 | AZI1     | 2.2                              | 1.8E-13              | azelaic acid induced 1   |
| AT1G27020 |          | 2.4                              | 1.9E-13              | Unknown  |
| AT2G14610 | PR1      | 2.3                              | 2.2E-13              | pathogenesis-related gene 1  |
| AT5G20790 |          | 2.8                              | 1.2E-12              | Unknown  |
| AT3G02040 | SRG3     | 2.1                              | 1.2E-12              | senescence-related gene 3  |

(Continued)

Table 2. (Continued)

| AGI locus | symbol    | fold change ( <i>esr1</i> -1/WT) | FDR-adjusted p-value | AGI description  |
|-----------|-----------|----------------------------------|----------------------|--|
| AT4G22470 |           | <b>3.7</b>                       | 1.7E-12              | protease inhibitor/seed storage/lipid transfer protein (LTP) family protein            |
| AT2G29900 | PS2       | 2.4                              | 9.0E-12              | Presenilin-2   |
| AT1G61120 | TPS04     | <b>7.2</b>                       | 1.8E-11              | terpene synthase 04  |
| AT4G25000 | AMY1      | <b>4.7</b>                       | 3.3E-11              | alpha-amylase-like   |
| AT3G44860 | FAMT      | <b>3.8</b>                       | 5.6E-11              | farnesoic acid carboxyl-O-methyltransferase  |
| AT5G10380 | RING1     | 2.7                              | 6.8E-11              | RING/U-box superfamily protein   |
| AT1G17710 |           | 2.6                              | 9.0E-11              | Pyridoxal phosphate phosphatase-related protein  |
| AT2G29350 | SAG13     | 2.4                              | 1.4E-10              | senescence-associated gene 13  |
| AT1G15520 | PDR12     | 2.6                              | 1.9E-10              | pleiotropic drug resistance 12   |
| AT5G10760 |           | 3.1                              | 3.7E-10              | Eukaryotic aspartyl protease family protein  |
| AT3G26830 | PAD3      | 2.2                              | 1.5E-09              | Cytochrome P450 superfamily protein, PHYTOALEXIN DEFICIENT 3                           |
| AT1G69880 | TH8       | <b>3.1</b>                       | 2.5E-09              | thioredoxin H-type 8   |
| AT3G25760 | AOC1      | 2.1                              | 2.9E-09              | allene oxide cyclase 1   |
| AT1G14250 |           | 2.5                              | 6.0E-09              | GDA1/CD39 nucleoside phosphatase family protein  |
| AT4G37990 | ELI3-2    | <b>6.3</b>                       | 7.5E-09              | elicitor-activated gene 3–2  |
| AT3G55970 | JRG21     | <b>4.1</b>                       | 1.5E-08              | jasmonate-regulated gene 21  |
| AT2G18660 | PNP-A     | <b>3.8</b>                       | 4.2E-08              | plant natriuretic peptide A  |
| AT3G17790 | PAP17     | 2.3                              | 4.4E-08              | purple acid phosphatase 17   |
| AT5G23980 | FRO4      | 2.6                              | 5.0E-08              | ferric reduction oxidase 4   |
| AT2G16005 |           | 2.2                              | 8.2E-08              | MD-2-related lipid recognition domain-containing protein                               |
| AT3G26840 |           | 2.3                              | 8.6E-08              | Esterase/lipase/thioesterase family protein  |
| AT3G28540 |           | 2.2                              | 1.3E-07              | P-loop containing nucleoside triphosphate hydrolases superfamily protein               |
| AT4G21830 | MSRB7     | 2.6                              | 2.5E-07              | methionine sulfoxide reductase B7  |
| AT2G14560 | LURP1     | 2.9                              | 2.5E-07              | LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA                            |
| AT1G10585 |           | <b>3.8</b>                       | 2.8E-07              | basic helix-loop-helix (bHLH) DNA-binding superfamily protein                          |
| AT2G39510 |           | 2.0                              | 3.0E-07              | nodulin MtN21 /EamA-like transporter family protein                                    |
| AT3G05630 | PLDP2     | 2.3                              | 3.0E-07              | phospholipase D P2   |
| AT3G46900 | COPT2     | 2.6                              | 3.9E-07              | copper transporter 2   |
| AT4G21326 | SBT3.12   | <b>3.1</b>                       | 4.6E-07              | subtilase 32   |
| AT2G44460 | BGLU28    | 2.4                              | 6.7E-07              | beta glucosidase 28  |
| AT5G42580 | CYP705A12 | 2.1                              | 7.2E-07              | cytochrome P450, family 705, subfamily A, polypeptide 12                               |
| AT5G23990 | FRO5      | <b>5.6</b>                       | 8.1E-07              | ferric reduction oxidase 5   |
| AT4G32810 | CCD8      | 2.9                              | 1.4E-06              | carotenoid cleavage dioxygenase 8  |
| AT5G20710 | BGAL7     | 2.0                              | 1.4E-06              | beta-galactosidase 7   |
| AT4G17470 |           | <b>3.6</b>                       | 2.0E-06              | alpha/beta-Hydrolases superfamily protein  |
| AT3G05400 |           | 2.0                              | 2.2E-06              | Major facilitator superfamily protein  |
| AT1G19380 |           | 2.8                              | 2.3E-06              | Protein of unknown function (DUF1195)  |
| AT1G32350 | AOX1D     | <b>3.1</b>                       | 2.9E-06              | alternative oxidase 1D   |
| AT5G04120 |           | 2.2                              | 5.8E-06              | Phosphoglycerate mutase family protein   |
| AT2G29470 | GSTU3     | <b>4.5</b>                       | 7.4E-06              | glutathione S-transferase tau 3  |
| AT3G22910 |           | 2.5                              | 8.6E-06              | ATPase E1–E2 type family protein / haloacid dehalogenase-like hydrolase family protein |
| AT1G76960 |           | 2.1                              | 1.2E-05              | Unknown  |
| AT4G21680 | NRT1.8    | <b>3.5</b>                       | 1.3E-05              | NITRATE TRANSPORTER 1.8  |
| AT5G45430 |           | 2.1                              | 1.5E-05              | Protein kinase superfamily protein   |

(Continued)



Table 2. (Continued)

| AGI locus | symbol  | fold change ( <i>esr1</i> -1/WT) | FDR-adjusted p-value | AGI description   |
|-----------|---------|----------------------------------|----------------------|---|
| AT3G45130 | LAS1    | <b>3.0</b>                       | 2.6E-05              | lanosterol synthase 1   |
| AT1G17420 | LOX3    | 2.3                              | 2.8E-05              | lipoxygenase 3  |
| AT2G43020 | PAO2    | <b>13.7</b>                      | 3.4E-05              | polyamine oxidase 2   |
| AT1G61800 | GPT2    | 2.0                              | 4.8E-05              | glucose-6-phosphate/phosphate translocator 2  |
| AT1G12030 |         | <b>3.3</b>                       | 5.6E-05              | Protein of unknown function (DUF506)  |
| AT1G02470 |         | <b>3.7</b>                       | 5.6E-05              | Polyketide cyclase/dehydrase and lipid transport superfamily protein                      |
| AT1G07620 | ATOBGM  | 2.3                              | 5.7E-05              | GTP-binding protein Obg/CgtA  |
| AT2G11810 | MGDC    | 2.3                              | 6.3E-05              | monogalactosyldiacylglycerol synthase type C  |
| AT5G08760 |         | 2.1                              | 6.9E-05              | Unknown   |
| AT1G07400 |         | 2.1                              | 7.4E-05              | HSP20-like chaperones superfamily protein   |
| AT1G60110 |         | <b>3.1</b>                       | 1.1E-04              | Mannose-binding lectin superfamily protein  |
| AT3G45860 | CRK4    | 2.1                              | 1.1E-04              | cysteine-rich RLK (RECEPTOR-like protein kinase) 4  |
| AT4G12490 |         | 2.2                              | 1.3E-04              | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein |
| AT4G11890 |         | 2.6                              | 1.8E-04              | Protein kinase superfamily protein  |
| AT4G04490 | CRK36   | 2.5                              | 2.3E-04              | cysteine-rich RLK (RECEPTOR-like protein kinase) 36                                       |
| AT5G24200 |         | 2.8                              | 2.6E-04              | alpha/beta-Hydrolases superfamily protein   |
| AT5G44420 | PDF1.2  | <b>3.6</b>                       | 2.6E-04              | plant defensin 1.2  |
| AT2G45130 | SPX3    | 2.8                              | 2.8E-04              | SPX domain gene 3   |
| AT2G04450 | NUDT6   | 2.2                              | 2.9E-04              | nudix hydrolase homolog 6   |
| AT4G24000 | CSLG2   | 2.8                              | 3.0E-04              | cellulose synthase like G2  |
| AT2G36970 |         | 2.1                              | 4.0E-04              | UDP-Glycosyltransferase superfamily protein   |
| AT2G26400 | ARD3    | <b>3.0</b>                       | 5.0E-04              | acireductone dioxygenase 3  |
| AT4G24340 |         | 2.5                              | 5.1E-04              | Phosphorylase superfamily protein   |
| AT5G39520 |         | <b>3.2</b>                       | 8.4E-04              | Protein of unknown function (DUF1997)   |
| AT1G05660 |         | 2.7                              | 8.6E-04              | Pectin lyase-like superfamily protein   |
| AT1G52100 |         | 2.5                              | 9.6E-04              | Mannose-binding lectin superfamily protein  |
| AT2G39040 |         | 2.5                              | 1.1E-03              | Peroxidase superfamily protein  |
| AT1G73805 |         | 2.1                              | 1.2E-03              | Calmodulin binding protein-like   |
| AT3G52970 | CYP76G1 | 2.3                              | 1.3E-03              | cytochrome P450, family 76, subfamily G, polypeptide 1                                    |
| AT4G36700 |         | 2.8                              | 1.4E-03              | RmlC-like cupins superfamily protein  |
| AT3G51450 |         | 2.1                              | 1.7E-03              | Calcium-dependent phosphotriesterase superfamily protein                                  |
| AT3G05650 | RLP32   | 2.0                              | 1.7E-03              | receptor like protein 32  |
| AT2G45570 | CYP76C2 | 2.2                              | 1.8E-03              | cytochrome P450, family 76, subfamily C, polypeptide 2                                    |
| AT1G71400 | RLP12   | 2.2                              | 1.9E-03              | receptor like protein 12  |
| AT2G22860 | PSK2    | <b>3.1</b>                       | 3.2E-03              | phytosulfokine 2 precursor  |
| AT4G21840 | MSRB8   | 2.3                              | 3.2E-03              | methionine sulfoxide reductase B8   |
| AT1G23730 | BCA3    | 2.6                              | 3.4E-03              | beta carbonic anhydrase 3   |
| AT5G46050 | PTR3    | 2.4                              | 3.5E-03              | peptide transporter 3   |
| AT3G43110 |         | 2.3                              | 3.6E-03              | Unknown   |
| AT2G40330 | PYL6    | 2.4                              | 4.3E-03              | PYR1-like 6   |
| AT5G52760 |         | 2.4                              | 4.8E-03              | Copper transport protein family   |
| AT1G15540 |         | 2.6                              | 4.9E-03              | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein                   |
| AT1G80160 |         | 2.1                              | 5.6E-03              | Lactoylglutathione lyase / glyoxalase I family protein                                    |
| AT1G51820 |         | 2.2                              | 5.9E-03              | Leucine-rich repeat protein kinase family protein   |
| AT3G59370 |         | 2.0                              | 6.0E-03              | Vacuolar calcium-binding protein-related  |

(Continued)

Table 2. (Continued)

| AGI locus | symbol   | fold change ( <i>esr1</i> -1/WT) | FDR-adjusted p-value | AGI description  |
|-----------|----------|----------------------------------|----------------------|--|
| AT3G09405 |          | 2.9                              | 6.2E-03              | Pectinacetylesterase family protein                                      |
| AT3G46660 | UGT76E12 | 2.1                              | 6.2E-03              | UDP-glucosyl transferase 76E12   |
| AT3G04530 | PPCK2    | 2.0                              | 6.5E-03              | phosphoenolpyruvate carboxylase kinase 2                                 |
| AT1G08310 |          | 2.1                              | 7.3E-03              | alpha/beta-Hydrolases superfamily protein                                |
| AT2G38240 |          | 2.9                              | 7.5E-03              | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein  |
| AT1G35230 | AGP5     | 2.3                              | 7.7E-03              | arabinogalactan protein 5  |
| AT2G34655 |          | 2.3                              | 8.8E-03              | Unknown  |
| AT3G13840 |          | 2.3                              | 9.0E-03              | GRAS family transcription factor   |
| AT2G26010 | PDF1.3   | 2.2                              | 9.1E-03              | plant defensin 1.3   |
| AT5G43690 |          | 2.9                              | 9.6E-03              | P-loop containing nucleoside triphosphate hydrolases superfamily protein |
| AT4G37700 |          | 2.3                              | 9.7E-03              | Unknown  |
| AT2G14210 | AGL44    | 2.9                              | 1.0E-02              | AGAMOUS-like 44  |
| AT3G06435 |          | 2.1                              | 1.0E-02              | Expressed protein  |
| AT5G14180 | MPL1     | 2.6                              | 1.1E-02              | Myzus persicae-induced lipase 1  |
| AT1G52890 | NAC019   | 2.7                              | 1.1E-02              | NAC domain containing protein 19   |
| AT1G19200 |          | 2.0                              | 1.2E-02              | Protein of unknown function (DUF581)                                     |
| AT3G57460 |          | 6.7                              | 1.2E-02              | catalytics;metal ion binding   |
| AT1G01680 | PUB54    | 2.1                              | 1.2E-02              | plant U-box 54   |
| AT1G33950 |          | 6.6                              | 1.3E-02              | Avirulence induced gene (AIG1) family protein                            |
| AT3G24310 | MYB305   | 2.5                              | 1.3E-02              | myb domain protein 305   |
| AT4G24110 |          | 2.1                              | 1.6E-02              | Unknown  |
| AT4G33560 |          | 2.0                              | 1.6E-02              | Wound-responsive family protein  |
| AT3G12070 | RGTB2    | 2.4                              | 1.8E-02              | RAB geranylgeranyl transferase beta subunit 2                            |
| AT1G73325 |          | 6.3                              | 1.8E-02              | Kunitz family trypsin and protease inhibitor protein                     |
| AT1G54020 |          | 5.2                              | 1.9E-02              | GDSL-like Lipase/Acylhydrolase superfamily protein                       |
| AT2G34350 |          | 2.7                              | 2.0E-02              | Nodulin-like / Major Facilitator Superfamily protein                     |
| AT3G46090 | ZAT7     | 3.1                              | 2.0E-02              | C2H2 and C2HC zinc fingers superfamily protein                           |
| AT2G32660 | RLP22    | 2.1                              | 2.1E-02              | receptor like protein 22   |
| AT5G27060 | RLP53    | 2.4                              | 2.2E-02              | receptor like protein 53   |
| AT4G27160 | SESA3    | 28.7                             | 2.3E-02              | seed storage albumin 3   |
| AT1G17380 | JAZ5     | 2.2                              | 2.4E-02              | jasmonate-zim-domain protein 5   |
| AT1G72260 | THI2.1   | 5.4                              | 2.4E-02              | thionin 2  |
| AT2G37740 | ZFP10    | 2.7                              | 2.4E-02              | zinc-finger protein 10   |
| AT1G32960 | SBT3.3   | 2.8                              | 2.5E-02              | Subtilase family protein   |
| AT1G71200 |          | 2.8                              | 2.7E-02              | basic helix-loop-helix (bHLH) DNA-binding superfamily protein            |
| AT1G65570 |          | 2.9                              | 2.8E-02              | Pectin lyase-like superfamily protein                                    |
| AT2G14620 | XTH10    | 2.1                              | 3.1E-02              | xyloglucan endotransglucosylase/hydrolase 10                             |
| AT1G36622 |          | 2.0                              | 3.1E-02              | Unknown  |
| AT1G19610 | PDF1.4   | 3.4                              | 3.2E-02              | Arabidopsis defensin-like protein  |
| AT1G28370 | ERF11    | 2.6                              | 3.2E-02              | ERF domain protein 11  |
| AT1G63055 |          | 7.9                              | 3.3E-02              | Unknown  |
| AT3G04510 | LSH2     | 2.9                              | 3.4E-02              | Protein of unknown function (DUF640)                                     |
| AT4G13410 | ATCSLA15 | 2.3                              | 3.6E-02              | Nucleotide-diphospho-sugar transferases superfamily protein              |
| AT5G44430 | PDF1.2c  | 12.8                             | 3.6E-02              | plant defensin 1.2C  |
| AT5G43290 | WRKY49   | 3.5                              | 3.9E-02              | WRKY DNA-binding protein 49  |

(Continued)

Table 2. (Continued)

| AGI locus | symbol | fold change ( <i>esr1-1</i> /WT) | FDR-adjusted p-value | AGI description   |
|-----------|--------|----------------------------------|----------------------|---|
| AT5G55410 |        | 3.7                              | 4.0E-02              | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein |
| AT4G01630 | EXPA17 | 2.1                              | 4.1E-02              | expansin A17  |
| AT2G45760 | BAP2   | 2.7                              | 4.1E-02              | BON association protein 2   |
| AT4G04500 | CRK37  | 2.0                              | 4.4E-02              | cysteine-rich RLK (RECEPTOR-like protein kinase) 37                                       |
| AT3G03480 | CHAT   | 3.5                              | 4.5E-02              | acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase   |
| AT4G28085 |        | 2.7                              | 4.7E-02              | Unknown   |
| AT1G69720 | HO3    | 3.4                              | 4.7E-02              | heme oxygenase 3  |
| AT5G46350 | WRKY8  | 2.1                              | 4.8E-02              | WRKY DNA-binding protein 8  |
| AT5G13220 | JAZ10  | 2.9                              | 4.9E-02              | jasmonate-zim-domain protein 10   |

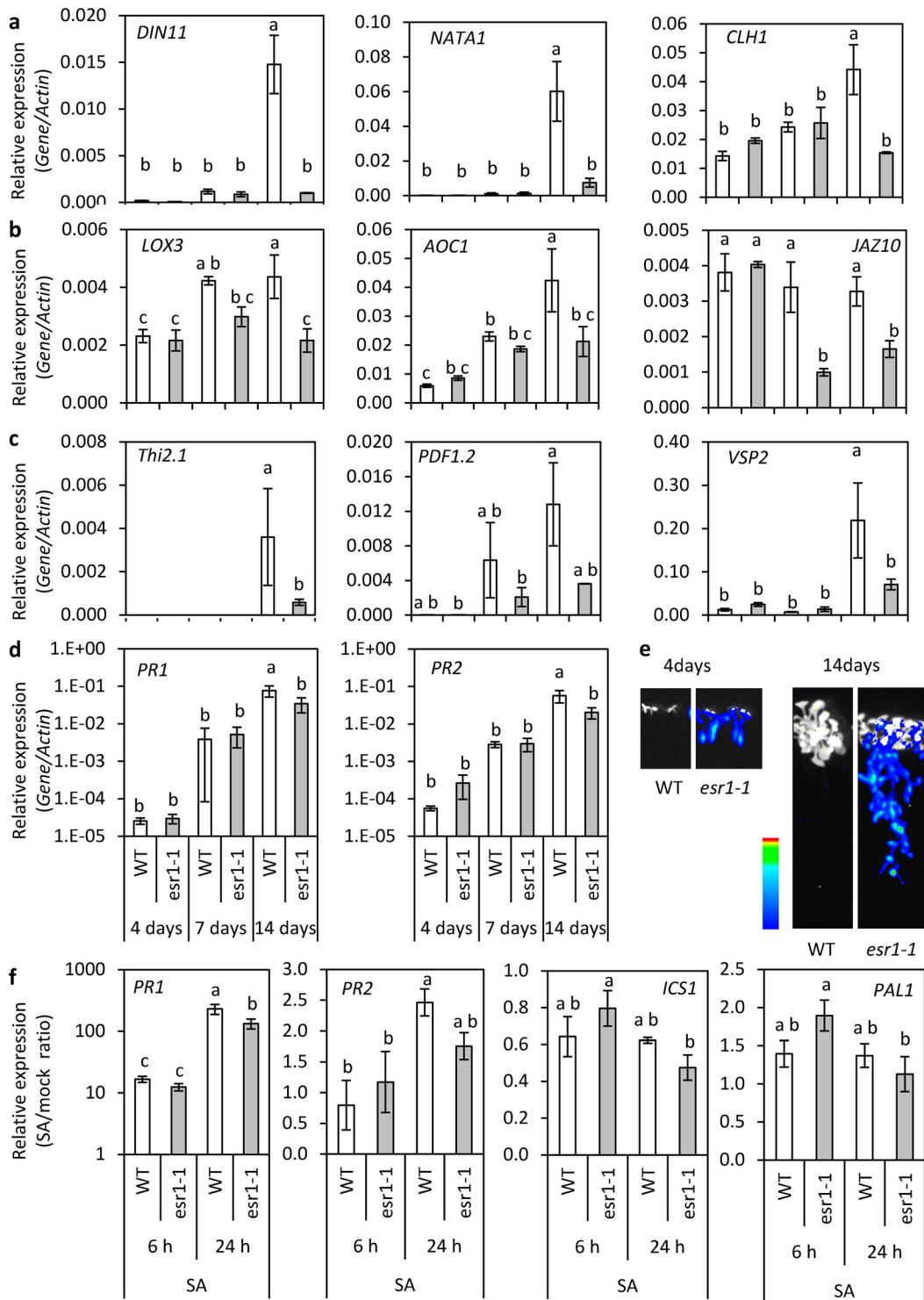
Fold change based on FPKMs from 3 biological replicates. Significance based on Benjamini-Hochberg correction for multiple-testing,  $P \leq 0.05$  adjusted by the False Discovery Rate. Values in bold are  $\geq 3$ -fold changes.

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enriched biological process GO terms were those involved in responses to stress, defense, biotic stimulus, other organisms, jasmonic acid (JA) including JA-biosynthesis and-signalling, fungus, wounding, SA, chemical stimulus and responses to starvation and nutrient levels (Fig 6e). As with the up-regulated *esr1-1* dataset, to confirm our RNAseq data we examined the expression of several down-regulated genes over a developmental time-course. This included the highly down-regulated genes *DARK INDUCIBLE 11 (DIN11)* encoding a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, *At2g39030/NATA1* encoding an Acyl-CoA N-acyl-transferase (NAT) superfamily protein with roles in pathogen resistance [60], as well as *CHLOROPHYLLASE 1/CORONATINE-INDUCED PROTEIN 1 (CLH1/COR11)* which has roles in several of the enriched biological process GO categories including defense responses, response to fungus and JA-signalling. No significant difference in *DIN11*, *NATA1* or *CLH1* expression was observed in 4- or 7-day old seedlings however, as in the RNAseq dataset they were highly down-regulated in 14-day old seedlings (Fig 7a).

### At5g53060/ESR1 affects basal JA-mediated responses involved in defense but not growth and development

A role for At5g53060 in JA-responses to our knowledge has not been described before, and as the down-regulated *esr1-1* gene list was enriched for genes with roles in these processes including defense and biotic stimulus (response to fungus and wounding), we were interested to dissect this further. We first examined the expression of representative JA-biosynthesis, signalling, and JA-regulated defense genes. Using qRT-PCR, the *LIPOXYGENASE 3 (LOX3)* and *ALLENE OXIDE CYCLASE 1 (AOC1)* genes involved in JA-biosynthesis, and *JASMONATE-ZIM-DO-MAIN PROTEIN 10 (JAZ10)* involved in repression of JA-responses were down-regulated in *esr1-1* in both 7- and 14-day old seedlings (Fig 7b) and were identified in the RNAseq dataset as down-regulated genes (Table 2). The down-regulation of these genes suggests an overall down-regulation of JA-signalling processes in *esr1-1* as their expression is in part regulated through JA-feedback loops [61–63]. In 14-day old seedlings the JA-regulated defense and wound marker genes analysed were all down-regulated in *esr1-1* compared to wild-type seedlings (Fig 7c). The expression of these marker genes in 4- or 7-day old seedlings was either lowly expressed or not detectable by qRT-PCR. Overall expression patterns in wild-type seedlings highlighted a trend in increasing expression from 4- to 14-days. Examination of these genes in publically



**Fig 7. Repression of JA-mediated gene expression in *esr1-1* increases with age.** (a-c) Expression of significantly up-regulated (a) novel RNA-seq identified, (b) JA-biosynthesis and signalling, (c) JA-regulated defense and wound-responsive genes, and (d) SA-regulated defense genes in *esr1-1* compared to wild-type (WT) seedlings as determined by qRT-PCR. Shown are values from 4, 7 and 14 day old seedlings (values are averages  $\pm$  SE of 3 biological replicates consisting of pools of 20 seedlings,  $P < 0.05$ , all pairs Student's t-test). Gene expression levels are relative to the internal control  $\beta$ -actin genes. (e) Increasing *GSTF8:LUC* activity in *esr1-1* seedlings during early development. (f) Fold changes in SA-marker genes in WT and *esr1-1* seedlings 6 and 24 hours post SA treatment. Shown are values from 12 day old seedlings (values are averages  $\pm$  SE of 3 biological replicates consisting of pools of 20–30 seedlings,  $P < 0.05$ , all pairs Student's t-test). Transcript levels of each gene of interest following SA treatment were normalised against the internal control  $\beta$ -actin genes and expressed relative to the normalised levels in mock-treated WT or *esr1-1* seedlings.

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available, developmental series transcriptome datasets (Genevestigator; [64]) also revealed similar gene expression profiles in wild-type plants (data not shown). *GSTF8:LUC* activity also increases in *esr1-1* seedlings over this timeframe (Fig 7e). Together, these results suggest At5g53060/ESR1 has a negative effect on *GSTF8:LUC* activity and a positive effect on the regulation of JA-mediated genes during early development.

In addition to roles in defense, JA also affects fertility, root growth and development [65–69]. However, neither *esr1-1* nor *esr1-2* are impaired in these processes (Fig 6a–6d). We also found the *esr1* mutants were not affected in JA-sensitivity as determined by methyl jasmonate (MeJA) root inhibition assays (S4 Fig). This suggests At5g53060/ESR1 functions in activation of a subset of JA-mediated responses.

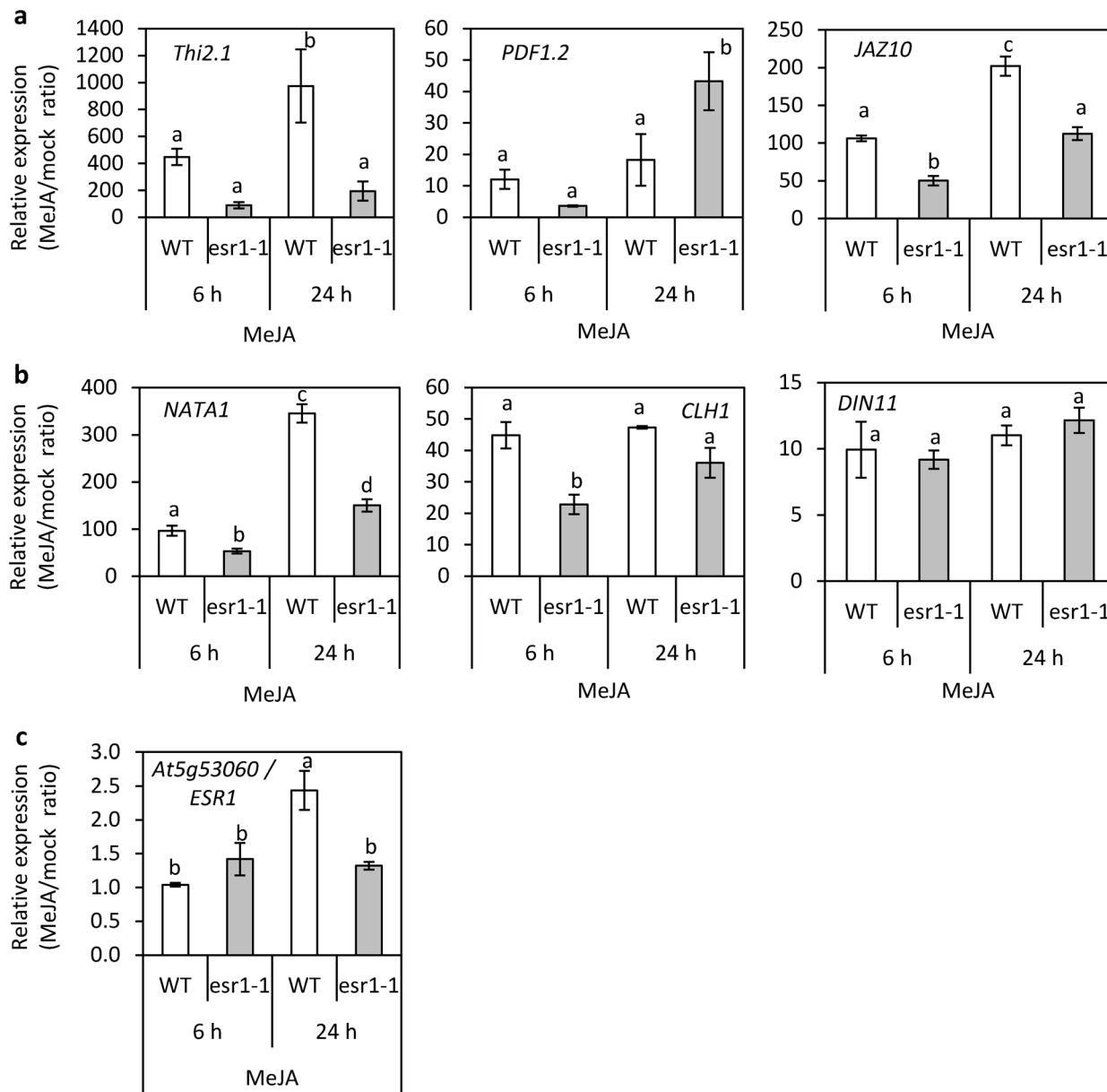
It is well known that antagonistic interactions occur between some aspects of JA and SA signalling (reviewed in [6, 8, 29, 70]). We therefore analysed expression of the SA-marker genes *PR1* and *PR2* in wild-type and *esr1-1* seedlings to determine if repression of JA-regulated genes in *esr1-1* was due to up-regulated SA-mediated signalling as is suggested by increased *GSTF8:LUC* activity and *GSTF8* expression in *esr1-1* following SA treatment (Fig 2). There was no significant difference in *PR1* or *PR2* expression between wild-type and *esr1-1* at 4 and 7 days of age, but their expression was significantly reduced in *esr1-1* at 14 days as was also detected by RNAseq (Fig 7d, Table 2). *PR1*, but not *PR2* expression, was also down-regulated in *esr1-1* following SA treatment (Fig 7f). To determine if other aspects of SA-signalling were altered in *esr1-1*, we determined expression of the *ISOCHORISMATE SYNTHASE1* (*ICS1*) and *PHENYLALANINE AMMONIA LYASE* (*PAL1*) genes involved in SA-biosynthesis (reviewed by [71]). Neither of these genes were significantly altered in expression suggesting ESR1 functions specifically in JA-signalling and down-regulation of *PR1* expression is due to non-SA-mediated processes.

### At5g53060/ESR1 is required for full activation of a subset of JA-regulated genes

Other mutants with reduced basal JA-biosynthesis or JA-regulated defense gene expression and exhibiting increased resistance to *Fusarium oxysporum* include *coi1* (*coronatine insensitive1*) and *pft1/med25* (*phytochrome and flowering time1*) [41, 45]. Expression of JA-regulated genes in these two mutants are also reduced following MeJA treatment. To determine if At5g53060/ESR1 affected the JA-inducibility of JA-regulated genes and other genes down-regulated in *esr1-1*, we examined the expression of *Thi2.1*, *PDF1.2*, *JAZ10*, *NATA1*, *CLH1* and *DIN11* in *esr1-1* and wild-type plants following MeJA or a mock treatment. As expected, MeJA treatment strongly induced *Thi2.1*, *PDF1.2* and *JAZ10* expression in wild-type plants relative to the mock-treated wild-type plants (Fig 8a). Expression of these genes was also induced by MeJA in *esr1-1* however, *Thi2.1* and *JAZ10* expression was 5-fold and 2-fold less respectively in *esr1-1* compared to wild-type levels at 6 and 12 hours post treatment. *PDF1.2* expression was also reduced in *esr1-1* at 6 hours but increased above wild-type levels at 24 hours. We next examined *NATA1*, *CLH1* and *DIN11* expression and found *esr1-1* had reduced induction of *NATA1* and *CLH1*, but did not affect the MeJA-induced expression of *DIN11* (Fig 8b). We also found *ESR1* expression is MeJA-inducible (Fig 8c). Combined, these results suggest ESR1 affects components of JA-signalling.

### Discussion

In a forward genetic screen using the defense and stress responsive *GSTF8* promoter, we isolated several alleles of the constitutive *GSTF8:LUC* expression mutant *esr1* encoding the KH-domain containing RNA-binding protein At5g53060. We identify At5g53060 as a susceptibility gene for *F. oxysporum* disease symptom development and a requirement for full-activation of



**Fig 8. *esr1-1* represses a subset of JA-induced gene expression.** (a-c) Fold changes in relative transcript abundance of (a) JA-regulated defense and signalling marker genes, (b) RNA-seq identified genes, and (c) *ESR1* in wild-type (WT) and *esr1-1* seedlings 6 and 24 hours post MeJA treatment. Shown are values from 12 day old seedlings (values are averages  $\pm$  SE of 3 biological replicates consisting of pools of 20–30 seedlings,  $P < 0.05$ , all pairs Student's t-test). Transcript levels of each gene of interest following MeJA treatment were normalised against the internal control  $\beta$ -actin genes and expressed relative to the normalised levels in mock-treated WT or *esr1-1* seedlings.

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components of JA-mediated gene expression. Four independent mutants of *At5g53060* termed *esr1-1*, *esr1-2*, *esr1-3* and *esr1-4* displayed increased resistance to *F. oxysporum* and define new roles for plant KH-domain containing proteins, linking *At5g53060* to biotic stress and JA-mediated defense responses.

In plants the most widely spread RNA-binding domains are the RNA Recognition Motif, the heterogeneous nuclear ribonucleoprotein K (hnRNP K) homology (KH), and Pentatricopeptide Repeat (PPR) [10, 72]. Most plant RNA-binding proteins contain one or more of these

domains, often combined with multiple auxiliary domains involved in protein-protein interactions or protein targeting, or other RNA-binding domains. An InterPro Scan of At5g53060 for known protein signatures only identified its five KH-domains (data not shown). KH-domain proteins typically contain more than one KH-domain where they can function independently or co-operatively to bind RNA or ssDNA [54]. The KH-domain was first identified in the human hnRNP K protein and is characterised by a conserved VIGXXGXXI sequence in the middle of the ~60 amino acid domain [11, 54]. In addition to At5g53060, only three other of the 26 predicted Arabidopsis KH proteins have been functionally characterised and these have roles in flowering, floral morphogenesis, and vegetative and reproductive development [73–75]. Unlike mutants of these KH genes, we found neither *esr1-1* nor *esr1-2* exhibited observable differences in flowering, growth or development (Fig 6a–6d).

In plants, RNA-binding proteins have been identified as regulators of floral transition, floral patterning, circadian rhythm, chromatin modification, ABA signalling and mediators of abiotic stress responses such as to dehydration, drought, flooding, salinity, cold and heat ([9, 10] and references within). However, few RNA-binding proteins have been characterized for roles in plant immunity [76, 77]. Examples include the RNA Recognition Motif containing proteins ATBRN1/ATRBP-DR1 and GLYCINE RICH PROTEIN 7 regulators of resistance against *Pseudomonas syringae* pv. *tomato* DC3000 [78, 79], and the double stranded RNA-binding domain proteins DICER LIKE2 and DICER LIKE4 involved in viral defense ([76, 80] and references within). Some RNA-binding proteins directly target pathogen RNA to control infection. For example PATHOGENESIS RELATED PROTEIN 10 members such as the cotton PR10 have ribonuclease activity [1, 81]. In addition to our findings on At5g53060, the only other plant KH-domain containing protein characterised for a role in plant immunity is BINDING TO TOMV RNA 1 (BTR1, At5g04430) [82] which functions by directly binding to Tomato Mosaic Virus (TOMV) RNA and preventing viral multiplication.

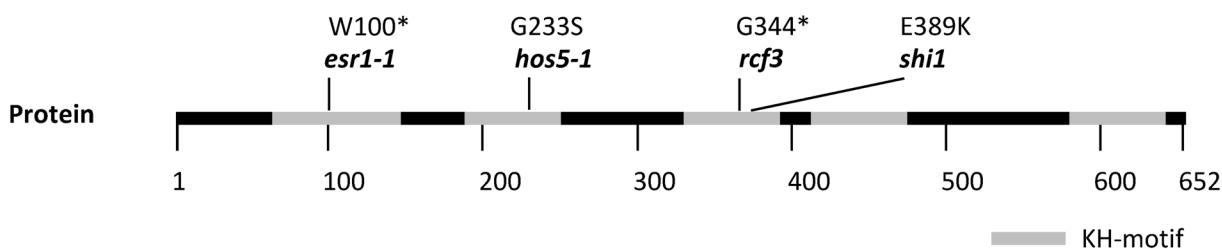
Through non-biased whole transcriptome sequencing we found *esr1-1* plants exhibited significant down-regulation of genes involved in responses to defense, biotic stimulus, fungus, wounding, and JA including JA-biosynthesis and-signalling (Fig 6). JA has roles in defense against specific microbial pathogens and insect pests, wounding responses, and roles in developmental processes such as fertility and root growth [65, 67–69]. The *esr1* mutants were not affected in latter responses but were affected in pathogen defense. Further, in addition to repression of basal JA-mediated gene expression, the MeJA inducibility of most JA-regulated genes tested were also repressed in *esr1-1* (Fig 8). Unlike the other repressed genes, *PDF1.2* expression in *esr1-1* was reduced at 6 hours but increased above wild-type levels at 24 hours. This suggests, in addition to activation of components of early JA-regulated gene expression, ESR1 may have roles in repression at later stages. We also found repression of JA-regulated genes in *esr1-1* was not due to antagonistic SA-JA crosstalk as SA-regulated marker genes in *esr1-1* showed no increase in their basal or SA induced levels (Fig 7).

The down-regulation of JA-signalling in *esr1-1* likely contributes to its enhanced resistance to *F. oxysporum* and increased susceptibility to *A. brassicicola* as this pathway confers susceptibility and resistance to these pathogens respectively. For example, the *coi1* and *pft1/med25* mutants, which are highly resistant to *F. oxysporum* and susceptible to *A. brassicicola*, also exhibit reduced expression of JA-biosynthesis (e.g. *LOX3*, *OPR*), JA-signalling (e.g. *JAZ9*, *JASMONIC ACID CARBOXYL METHYLTRANSFERASE*) and JA-regulated defense/senescence associated genes (e.g. *Thi2.1*, *PDF1.2*, *CLH1*) under mock, *F. oxysporum* or MeJA induced conditions [41, 45, 51]. While defensive components of JA-signalling do contribute positively to *F. oxysporum* resistance (e.g. increased *PDF1.2*, *Thi2.1* expression), global up-regulation of JA-signalling including its non-defensive components promote susceptibility [31, 44, 46, 83]. For example, senescence is proposed to strongly contribute to *F. oxysporum* disease symptom development

[41]. Unlike the *coi1* and *pft1* mutants which have impairments in growth or development (*coi1* is male sterile with insensitivity to MeJA inhibition of root growth, while *pft1* is delayed in flowering [45, 84]), *esr1-1* is indistinguishable from wild-type plants (Fig 6a–6d and S4 Fig). We also inoculated *esr1-1* with another root-infecting necrotrophic fungal pathogen, *R. solani* (isolates AG8 or AG2-1) but found no difference in disease phenotypes compared to wild-type plants (Fig 3a). It is suggested that neither JA or SA signalling pathways contribute to *R. solani* resistance or susceptibility in Arabidopsis [42].

Through independent forward genetic screens utilizing abiotic stress inducible promoters, At5g53060 was shown to be nuclear localised and have roles in diverse transcriptional processes including mRNA capping efficiency, polyadenylation site selection, mRNA export, and in the regulation of expression and alternate splicing of some stress inducible genes ([26, 27, 56] this work). Some of these processes, including its nuclear localisation, are mediated through At5g53060 interactions with the RNA PolII CTD interacting phosphatase protein CPL1 [25, 27, 56, 59] whose expression is up-regulated in *esr1-1* (Table 1, S3 Fig). We also tested the expression of *CPL1* and other genes up-regulated in *esr1-1* (*SYTB*, *STP11*, *At3g54160*) for responsiveness to SA or MeJA and found little change (S5 Fig) suggesting they may be regulated at the post transcriptional level by ESR1 or through other signalling pathways.

We characterised four mutants of At5g53060. The *esr1-1* mutant confers a Tryptophan to STOP codon substitution (W100\*) in the first of five At5g53060 KH-domains, *esr1-2* is a null T-DNA insertional inactivation line, while *esr1-3* and *esr1-4* harbour mutations at splice site junctions (Figs 4 and 5). Three other independently identified At5g53060 alleles confer other mutations. The *rcf3-1* (*regulator of CBF gene expression 1*) mutant isolated through a cold responsive *CBF2* promoter screen confers a Glycine to STOP codon substitution (G344\*) within the third At5g53060 KH-domain and displays increased heat tolerance [26]. The *shi1* (*shiny1*) mutant isolated through the salt inducible sulfotransferase *AtSOT12* promoter confers a Glutamic Acid to Lysine substitution (E389K) also within the third KH-domain [27]. The *shi1* mutant is more resistant to ABA during germination and has increased sensitivity to cold stress [27]. Unlike these mutants of At5g53060, the *hos5-1* mutant has increased sensitivity to ABA and salt stress, although tolerance/sensitivity to other abiotic stresses has not yet been tested for this At5g53060 mutant allele [56]. The *hos5-1* (*high osmotic stress gene expression 5*) mutation confers a Glycine to Serine change (G233S) within the second At5g53060 KH-domain. Interestingly, *esr1-1*, *rcf3-1* and *shi1* mutations (Fig 9) disrupt either the first or third KH-domains, both of which along with the fourth domain can interact with CPL1 ([27, 56, 59] this work). Although the *shi1* mutant confers only an amino acid substitution, this change disrupts the CPL1 interaction [27]. The second KH-domain and location of the *hos5-1* mutation (Fig 9) does not interact with CPL1 but may affect RNA binding [56]. This may explain why *hos5-1* is more sensitive to ABA while *shi1* is more tolerant. As with our *esr1-1* and *esr1-2* findings, *rcf3-1* and *shi1* exhibit increased expression of their *Promoter:LUC* transgenes but not of their



**Fig 9. Location and effect of At5g53060/ESR1/RCF3/SHI1/HOS5 mutations.** At5g53060 domain structure with the position and predicted nature of published mutations indicated relative to the first methionine.

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endogenous stress-inducible genes under basal conditions [26, 27]. We did however find *GSTF8* expression was significantly higher than wild-type in *esr1-1* following SA treatment (Fig 2b) suggesting regulation of *GSTF8:LUC* promoter and endogenous *GSTF8* differ under basal conditions. Indeed, [27] suggest under basal conditions the ESR1/SHI1-CPL1 complex may associate with other repressors on general transcriptional machinery targeting stress responsive promoters and upon stress inducing conditions this complex is modified.

In summary, we identified roles for the KH-domain RNA-binding protein At5g53060 in JA- and biotic induced stress responses, and define new functions for KH-domain proteins in plants. Further research of interest will be determining At5g53060/ESR1 direct RNA targets, which are yet to be identified, and other proteins it interacts with under specific biotic stresses, in particular those with JA-involvement.

## Supporting Information

**S1 Fig. Next Generation Mapping locates the *esr1-3* and *esr1-4* loci.** (a-b) Whole-genome sequencing of homozygous (a) *esr1-3* or (b) *esr1-4* F<sub>2</sub>s from *esr1* and Ler outcrosses coupled with the Next Generation Mapping tool identifies SNP deserts (underlined region) corresponding to linkage to the *esr1* mutations.

(TIF)

**S2 Fig. *esr1* mutants have altered thermo-tolerance.** (a) *esr1* mutants are more tolerant of heat stress as measured by the proportion of leaf area non-bleached. Seedlings were grown on MS agar plates for 7 days at 21°C, treated at 21°C (control) or 45°C (heat) for 90 minutes, then returned to 21°C for 4 days followed by measurement of bleached area. Values are averages ± SE (n = 10). Asterisks indicate values that are significantly different (\*\**P*<0.01 Student's *t*-test) from wild-type (WT). Similar results were obtained in independent experiments. (b) Average *GSTF8:LUC* expression per WT and *esr1-1* seedling per hour after treatment with heat (45°C) or control treatment (21°C). Values are averages ± SE (n = 5) from 7 day old seedlings.

(TIF)

**S3 Fig. Confirmation of *esr1-1* up-regulated genes by qRT-PCR.** Expression confirmation of subset of significantly up-regulated genes in *esr1-1* compared to wild-type (WT) seedlings. Shown are values from 4, 7 and 14 day old seedlings (values are averages ± SE of 3 biological replicates consisting of pools of 20 seedlings, *P*<0.05, all pairs Student's *t*-test). Gene expression levels are relative to the internal control *β-actin* genes.

(TIF)

**S4 Fig. *esr1* mutants do not have altered root sensitivity to JA.** Sensitivity of wild-type (WT), *esr1-1* and *esr1-2* seedlings to JA was determined by MeJA inhibition of root growth on control media or media containing 25 μM or 50 μM MeJA. Root elongation of each line when grown on MeJA media was calculated as a percentage relative to their root length on the control. Values are average ± SE for 5 biological replicates consisting of pools of 10 seedlings; *P*<0.05, all pairs Student's *t*-test). Similar results were obtained in an independent experiment.

(TIF)

**S5 Fig. Basal *esr1-1* up-regulated genes are similarly expressed in wild-type and *esr1-1* when treated with MeJA or SA.** Fold changes in relative transcript abundance of RNA-seq identified genes in wild-type (WT) and *esr1-1* seedlings 6 and 24 hours post MeJA or SA treatment. Shown are values from 12 day old seedlings (values are averages ± SE of 3 biological replicates consisting of pools of 20–30 seedlings). Transcript levels of each gene of interest following MeJA or SA treatment were normalised against the internal control *β-actin* genes and expressed

relative to the normalised levels in mock-treated WT or *esr1-1* seedlings. The numbers on each bar show fold increase or fold decrease caused by each treatment relative to mock-treated plants. *PDF1.2* and *PR1* were used as marker genes for MeJA and SA treatment respectively. (TIF)

**S1 Table. Primers used for construct generation, mapping and qRT-PCR.**

(XLSX)

**S2 Table. *esr1-1* significantly down-regulated genes.**

(XLSX)

**S3 Table. *esr1-1* significantly up-regulated genes.**

(XLSX)

**S4 Table. Gene Ontology (GO) terms significantly over-represented in genes down-regulated in *esr1-1* versus wild-type.**

(XLSX)

**S5 Table. Gene Ontology (GO) terms significantly over-represented in genes up-regulated in *esr1-1* versus wild-type.**

(XLSX)

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## Author Contributions

Conceived and designed the experiments: LFT KBS. Performed the experiments: LFT LGK LOS. Analyzed the data: LFT JKH LOS. Contributed reagents/materials/analysis tools: JKH KBS. Wrote the paper: LFT LGK JKH LOS KBS.

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