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Jasmonic acid-dependent regulation of seed dormancy following maternal herbivory in Arabidopsis

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Summary

- Maternal experience of abiotic environmental factors such as temperature and light are well-known to control seed dormancy in many plant species. Maternal biotic stress alters offspring defence phenotypes, but whether it also affects seed dormancy is remains unexplored.
- We exposed *Arabidopsis thaliana* plants to herbivory and investigated plasticity in germination and defence phenotypes in their offspring, along with the roles of phytohormone signalling in regulating maternal effects.
- Maternal herbivory resulted in the accumulation of jasmonic acid-isoleucine and loss of dormancy in seeds of stressed plants. Dormancy was also reduced by engineering seed-specific accumulation of jasmonic acid in transgenic plants. Loss of dormancy was dependent on an intact jasmonate signalling pathway and was associated with increased gibberellin content and reduced abscisic acid-sensitivity during germination. Altered dormancy was only observed in the first generation following herbivory, whereas defence priming was maintained for at least two generations.
- Herbivory generates a jasmonic acid-dependent reduction in seed dormancy, mediated by alteration of gibberellin and abscisic acid signalling. This is a direct maternal effect, operating independently from transgenerational herbivore resistance priming.

Key Words: Abscisic acid; *Arabidopsis thaliana*; Defence; Dormancy; Herbivory; Jasmonic acid; Priming; Seeds

Introduction

Phenotypic plasticity provides plants with the ability to maximise their lifetime reproductive fitness in changing environments. Examples include developmental plasticity, such as photomorphogenic responses, and physiological plasticity, such as inducible defences against pests and pathogens. Such responses can affect not only the plant perceiving an environmental signal, but also its offspring (Roach & Wulff, 1987). For example, seed dormancy, a key adaptive trait that links plant life history to seasonal change, is determined by maternal responses to day length and temperature in many species (Fenner, 1991; Donohue, 2009; Kendall & Penfield, 2012). More recently, evidence has accumulated for parental effects on a wider range of offspring traits, including responses to biotic and abiotic stresses (Holeski *et al*., 2012; Tricker, 2015). Examples include changes in trichome density in response to wounding (Holeski, 2007), altered drought tolerance and stomatal development in response to water stress (Sultan et al., 2009; Tricker *et al*., 2013), and enhanced resistance to herbivory and disease (Luna *et al*., 2012; Rasmann *et al*., 2012). Transgenerational phenotypic plasticity can arise from direct maternal effects on developing seeds (*e.g*. altered seed size/nutrient allocation, transfer of stress signals to the embryo), but in some instances, phenotypes are transmitted through one or more stress-free generations (Luna *et al*., 2012). Transgenerational effects that persist across multiple generations are unlikely to be simple maternal effects, but have instead been linked with epigenetic mechanisms for 'soft inheritance' of adaptive traits (Holeski *et al*., 2012; Tricker, 2015).

The mechanisms underlying the establishment of seed dormancy in response to the maternal environment are still poorly defined, but plant hormones are recognised as important regulators of dormancy and germination. Abscisic acid (ABA) and gibberellic acid (GA) in particular play central, but antagonistic roles. ABA is essential for the imposition and maintenance of dormancy, and inhibits germination, whilst GA promotes germination (Holdsworth *et al*., 2008; Graeber *et al*., 2012). Hormones are also central to the regulation of plant defence. Salicylic acid (SA) and jasmonic acid (JA) are primary regulators of disease and herbivore resistance responses, and interactions with other hormones such as ethylene, ABA, GA and auxins serve to fine tune defences, depending upon the nature of the attacker (Robert-Seilaniantz *et al*., 2011; Pieterse *et al*., 2012). In addition to directly activating inducible defences in response to herbivore and pathogen attack, the JA and SA pathways also function to establish priming – that is, a state in which plant tissues become sensitised to future attack, permitting enhanced defence in response to subsequent infection (Conrath *et al*., 2015). Transgenerational priming of plant defences also requires functional SA and JA signalling pathways (Luna *et al*., 2012; Rasmann *et al*., 2012).

Whilst the functions of ABA and GA in seed dormancy and germination have been well-characterised, the roles of other hormones, including JA, are poorly understood (Linkies & Leubner-Metzger, 2012; Wasternack *et al*., 2013). Although effects on dormancy have rarely been reported in mutants in JA biosynthesis or signalling, accumulation of the JA precursor 12-oxo-phytodienoic acid (OPDA), causes increased seed dormancy in the *comatose* (*cts*) mutant of Arabidopsis in a mechanism involving ABA and several other dormancy-promoting factors (Dave *et al*., 2011; 2016). Addition of exogenous JA to mature seeds has been found to inhibit germination in several species (Wilen *et al*., 1991; Ellis & Turner, 2002; Worrall *et al*., 2012). In contrast, seeds of the Arabidopsis mutants *jar1-1* and *coi1-16* (deficient in the biosynthesis and perception of the major bioactive jasmonate, jasmonoyl-L-isoleucine (JA-Ile) respectively), are hypersensitive to ABA in germination assays (Staswick *et al*., 1992; Ellis & Turner, 2002), indicating a role for endogenous JA in promoting germination. Consistent with this, seeds of the relatively non-dormant Col-0 Arabidopsis ecotype contained 10-fold higher JA levels than seeds of the highly dormant Cvi ecotype (Preston *et al*., 2009). Members of the jasmonate family of hormones may therefore play multiple roles in seed dormancy and germination.

Rapid production of jasmonates is a stereotypical response to wounding and herbivory in many plant species, including Arabidopsis (Howe & Jander, 2008). Both JA and its precursor OPDA accumulate in damaged tissues (Koo *et al*., 2009), and each is responsible for the regulation of distinct transcriptional responses (Taki et al., 2005). Since various roles for OPDA and jasmonic acid in seed development and germination have been suggested (Dave *et al*., 2011; Linkies & Leubner-Metzger, 2012; Wasternack *et al*., 2013; Dave *et al*., 2016), we were interested to test whether herbivory affected seed germination in Arabidopsis. Our results indicate that in addition to effects on offspring defence responses, exposure of maternal plants to herbivory has a major impact on seed dormancy. The effect on dormancy is distinct from transgenerational defence priming responses, and is a consequence of the exposure of developing seeds to elevated levels of jasmonic acid and resultant changes in the sensitivity of seed germination to ABA.

Materials and Methods

Plant materials and plant growth

The *Arabidopsis thaliana* (L.) Columbia-0 (Col-0) accession and *jar1-1* and *jin1-9* mutants were originally obtained from the Nottingham Arabidopsis Stock Centre, UK. Seeds were stratified on ½ MS agar with 1% sucrose at 4°C in the dark for 2 d before transfer to a plant growth chamber

(Percival Scientific, Iowa, USA) set at 22 ± 1°C with a 10 h light cycle provided by Osram fluora lamps delivering 100 ± 20 µmol m^{-2} s^{-1}. After 10 days, seedlings were transferred to a sand: compost mixture (1:4) and cultivated in a controlled environment growth room (10:14 h light:dark; 20.5°C:18.5°C day:night temperature; 55% relative humidity) under Philips TLD840 fluorescent lamps delivering 250 ± 15 µmol m^{-2} s^{-1} at bench height. There were no nutrient additions during the course of the life of the plants.

For parental herbivore treatments, Arabidopsis Col-0 plants were grown in 15 cm square pots (9 seedlings per pot) in a Sanyo MLR-350 controlled environment chamber. Plants were infested with red spider mite (*Tetranychus urticae* Koch) by transferring infested leaves from bean plants carrying stock cultures of mites to pots when plants were at the rosette stage just prior to bolting. Plants were then left to set seed in the presence of herbivores. Control plants were grown under identical conditions in a matched growth chamber in the absence of herbivores. Three independent experiments were performed, each including 3 pots per treatment. For simulated herbivory, the rosette leaves of 5-week-old plants were wounded, once per leaf, using blunt forceps to crush the leaf blade across the lamina, perpendicular to the main vein. The majority of rosette leaves were wounded, three or four leaves per day, over a period of several days until the first bolt appeared.

To produce transgenic *OLE2::AOS* lines, the *OLE2* promotor (proOLE2) and *AOS* coding DNA sequence (cds) were PCR-amplified using the primer pairs Ole2-Pro-EcoRI-Fwd/Ole2-Pro-NcoI-Rev and AOS-OE-NcoI-Fwd/ AOS-OE-XmaI-Rev (For primer sequences see Supporting Information Table S1). The original CaMV 35S promoter of the pFGC5941 binary vector was replaced with proOLE2 using the EcoRI and NcoI restriction enzymes sites to give rise to pFGC5941-proOLE2. The AOS cds was subsequently cloned into pFGC5941-proOLE2 using the NcoI and XmaI sites. The resulting construct was introduced into Arabidopsis Col-0 via Agrobacterium-mediated transformation.

Germination assays

Dormancy assays were performed on freshly-harvested seed. To ensure consistent collection of newly-matured seed, plants were gently shaken to remove seed from dry, dehiscent siliques prior to harvesting seeds for analysis. Three to five days later, seed were collected from recently-matured siliques by gently agitating inflorescences. Seeds were then sterilised and plated on solid medium composed of half-strength MS basal salts, 1% (w/v) sucrose. Plates were incubated in a plant growth chamber as described above. Germination was scored by counting seeds with an emerged radicle. Seed lots to be compared were harvested on the same day from individual plants grown under identical environmental conditions. To measure ABA and NaCl sensitivity, after-ripened seeds, which

had been stored at room temperature in the dark, were plated on agar medium composed of MS basal salts, 1% (w/v) sucrose, with the inclusion of 2.5 µM ABA or 200 mM NaCl as appropriate. Seed were cold-stratified at 4°C for 2 d to break dormancy prior to transfer to the growth cabinet.

Assays for herbivore and pathogen resistance

Cultures of red spider mite were maintained on French bean plants before the experiments. Adult mites were collected from stock plants and released onto leaves (5 mites per plant) of Arabidopsis plants grown in controlled environments under standard conditions. Mites were allowed to feed for 9 d. The extent of plant-mediated resistance to *T. urticae* was measured by counting the number of mite eggs present using a binocular microscope. Stock cultures of the green peach aphid, *Myzus persicae* (Sulzer), were maintained on Col-0 Arabidopsis plants prior to experiments. Three adult females were transferred to experimental plants and populations measured 7 days later by counting aphids of all developmental stages present on the plant.

Pseudomonas syringae resistance assays were performed with a bioluminescent *luxCDABE*-tagged strain (*Pst* DC3000-*lux*; Fan *et al*., 2008). *Pst* DC3000-*lux* bacteria were cultivated at 28°C and 180 rpm in King's B medium containing rifampicin and kanamycin. Five-week-old plants were sprayed with a bacterial suspension containing 10^8 colony-forming units mL^{-1} in 10 mM $MgSO_4$ and 0.05% (v/v) Silwet L-77. A fine mist was used to spray leaves until imminent runoff. After inoculation, plants were kept at 100% relative humidity. For bacterial quantification, samples comprising nine leaf discs (three leaf discs per plant taken from three different plants and pooled) were homogenized in 1 ml 10 mM $MgCl_2$. After brief centrifugation, the intensity of bioluminescence of a 250 µl aliquot of bacterial suspension was measured using a filter-based luminescence microplate reader (FLUOstar Omega, BMG Labtech, Germany) at maximum sensitivity.

Hormone determination

Phytohormone determinations from dry seed were performed as previously described (Dave *et al*., 2011).

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Arabidopsis leaves were wounded across the lamina using a haemostat. Leaves were flash frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle and liquid nitrogen. Total RNA was extracted and purified using the RNeasy Plant Mini Kit (Qiagen, Germany). Complementary DNA

was synthesized from 2 µg of total RNA using oligo(dT) primer (Supplementary Table 1) and SuperScript II Reverse Transcriptase (Invitrogen, UK). SYBR Green Jump Start Taq ReadyMix (Sigma, UK) and the Agilent Technologies Stratagene Mx3005P QPCR System were employed for quantitative real-time PCR analysis. The thermal cycling program was composed of an initial 3 min at 95°C for 10 min, followed by 40 two-step cycles at 95°C for 30 s and 60°C for 1 min. The melting curve was used to verify single amplicon. Normalization of gene expression across different samples was performed with actin (*ACT2*) as an internal control. Relative expression levels at each time point were determined using Agilent Technologies MxPro software using the delta C_t method. All gene-specific primers used for real-time PCR are listed in Supporting Information Table S1.

Statistics

Data were compared using either Student's *t*-test, a Mann-Whitney test, one-way or two-way ANOVA in GraphPad Prism software, version 4.03. Prior to statistical analysis, the distributions and variances of data were tested using the D'Agostino & Pearson omnibus normality test and an *F* test respectively, in GraphPad Prism software, version 4.03. Where data were not normally distributed, the appropriate non-parametric test was applied. Where variances were not equal between data sets used in *t*-tests, Welch's correction was applied. Germination time series data were analysed using a 2x2 factorial repeated-measures ANOVA general linear model in the IBM SPSS Statistics package.

The underlying data in this paper is available from https://dx.doi.org/10.17635/lancaster/researchdata/129.

Results

Maternal herbivory reduces seed dormancy via changes in seed hormone content and sensitivity

We used red spider mite (*Tetranychus urticae*), a cell content-feeding arachnid herbivore that activates JA-dependent defences (Martel *et al*., 2015), to infest Arabidopsis plants allowed them to set seed. Spider mite-infested plants will hereafter be referred to as the SM_0 generation and their progeny as the SM_1. Control C_1 seed were collected from a corresponding C_0 parental generation grown in parallel with the SM_0. Freshly-harvested seed were sown on agar to investigate the degree of dormancy in C_1 and SM_1 lines. We consistently observed dramatic differences in germination between treatments. C_1 seed were relatively dormant, exhibiting around 20-30% germination after 7 days. In contrast, SM_1 seed collected from plants exposed to herbivory displayed very low levels of

dormancy, with seeds exhibiting rapid germination, and reaching 80-90% germination after 7 days (Fig. 1a). We also measured dormancy in C_2 and SM_2 seed collected from C_1 and SM_1 parents grown under stress-free conditions. C_2 and SM_2 seed showed similar levels of dormancy to one another (Fig. 1b), indicating that the reduced dormancy phenotype is restricted to seed produced by plants which have directly suffered herbivory and is not transmitted to subsequent generations.

To examine the role of JA signalling in the regulation of dormancy in the absence of biotic stress, we tested germination in freshly-harvested seed of the JA-Ile-deficient *jar1-1* mutant (Staswick *et al*., 1992), and in the JA signalling mutant *jin1-9*, which lacks the JA master-regulator protein, MYC2 (Anderson *et al*., 2004). Both mutants exhibited increased dormancy relative to seeds from the wild-type Col-0 background (Fig. 1c), suggesting that JA-Ile is a negative regulator of dormancy. We next addressed the question of whether the effect of herbivory on seed dormancy is JA-Ile-dependent. JA mutants are typically highly susceptible to herbivory, which presents a major confounding factor in experiments attempting to measure effects of herbivory on seed dormancy. We therefore tested whether simulation of herbivory by mechanical wounding, a common proxy for herbivore damage that also activates the JA pathway, would produce similar effects on dormancy as spider mite feeding. Experiments in Col-0 plants confirmed that wounding rosette leaves prior to or during flowering reduced primary dormancy similar to herbivory (Supporting Information, Fig. S1). We then tested the effect of simulated herbivory on seed dormancy in the *jar1-1* mutant and in the JA receptor mutant *coi1-16*. *coi1-16* is a weak *coi1* allele that retains male fertility, therefore allowing collection of seed from these plants (Ellis & Turner, 2002). In both the respective wild-type backgrounds, simulated herbivory significantly reduced dormancy, whereas it had no effect in the *coi1-16* and *jar1-1* mutants (Fig. 1d).

To further investigate possible mechanisms underlying the loss of dormancy following herbivory, we measured the levels of relevant phytohormones in C_1 and SM_1 seed. We found that the amounts of both JA and JA-Ile were significantly elevated in SM_1 seed compared to C_1 seed from unstressed parents (Fig. 2a, b). The amounts of OPDA in dry seed were not significantly different, however (Fig. 2c). Consistent with the antagonistic roles of ABA and GA in promoting dormancy and germination respectively, we found that compared with control C_1 seed, SM_1 seed contained significantly lower levels of ABA, but elevated levels of GA_4 (Fig. 2d, e).

Because the balance between dormancy and germination is affected not only by the levels of ABA and GA, but also by sensitivity to these hormones, we tested the sensitivity of SM_1 and SM_2 seed to ABA. Germination of C_1 seed was prevented by the presence of ABA in the medium, whilst in contrast, germination of SM_1 seed was not affected (Fig. 3a). ABA sensitivity was restored in SM_2 seed, however (Supporting Information Fig. S2a). The effect of herbivory on ABA sensitivity is

consistent with the observation that seeds of the *jar1-1* and *coi1-16* mutants, which are impaired in JA signalling, are hypersensitive to ABA (Staswick *et al*., 1992; Ellis & Turner, 2002). ABA is also important in controlling germination under abiotic stress conditions (Ruggiero *et al*., 2004). We therefore examined the effect of including 200 mM NaCl in the germination medium. Exposure to salt stress strongly inhibited germination of C_1 seed, but germination of SM_1 seed was completely insensitive to NaCl (Fig. 3b). Investigation of the effects of salinity on post-germination seedling growth revealed that the inhibition of primary root growth by NaCl was also lost in SM_1 lines (Supporting Information Fig. S2b).

Spider mite herbivory establishes primed JA-dependent resistance responses that are maintained for at least two generations

In addition to seed traits, we also examined the impact of herbivory on defence phenotypes in the progeny of mite-infested plants. Compared to control C_1 plants, SM_1 lines supported a lower reproductive capacity (egg laying) of *T. urticae* (Fig. 4a) and smaller populations of the generalist aphid herbivore, *Myzus persicae* (Fig. 4b), indicating enhanced JA-dependent herbivore resistance responses. We also assessed SA-dependent resistance to the bacterial pathogen, *P. syringae* in SM_1 lines primed by parental spider mite infestation. As shown in Fig. 4c, SM_1 lines were more susceptible to infection than C_1 control lines.

To investigate the persistence of these transgenerational priming phenotypes, we also tested JA- and SA-dependent defence responses in SM_2 and C_2 seed collected from SM_1 and C_1 lines grown under stress-free conditions. The results presented in Figs 4d and 4e, show that increased herbivore resistance and pathogen susceptibility are maintained for at least two generations following the initial imposition of herbivore stress.

Elevated seed jasmonates are sufficient to cause changes in dormancy and defence priming

To test whether elevated seed jasmonate content is sufficient for the effect on dormancy that we observe following parental herbivory, we produced transgenic lines in which the JA biosynthetic enzyme allene oxide synthase (*AOS*), is over-expressed under the control of the promoter from the seed development-specific oleosin gene, *OLE2* (Miquel *et al*. 2014). Two transgenic lines named *OLE2::AOS* 12-2 and *OLE2::AOS* 17-4, which expressed high levels of *AOS* mRNA in seeds (Supporting Information Fig. S3), were selected for further analysis. When we assayed dormancy in fresh seed collected from *OLE2::AOS* lines grown in the absence of herbivory, they exhibited significantly higher

germination frequencies than non-transformed controls (Fig. 5a). Hormone profiling of seed from the *OLE2::AOS* transgenic lines shows that similar to seed from mite-infested plants, they contain ~50% more JA-Ile than controls (Fig. 5c), and significantly elevated levels of GA_4 (Fig. 5f). The levels of OPDA and ABA were not affected in the transgenic lines (Fig. 5d, e). Although seed from these plants did not show altered ABA levels, when we assayed germination on media supplemented with ABA, ABA sensitivity was reduced (Fig. 5b), consistent with the phenotype of seed from herbivore-infested parents.

We were also interested to see whether seed accumulation of JA-Ile in the *OLE2::AOS* transgenic lines affected defence responses in plants grown from these seed. Assays of spider mite reproductive performance revealed a significant increase in herbivore resistance in the transgenic plants (Fig. 6a), whereas resistance to *P. syringae* was significantly lower than in Col-0 controls (Fig. 6b). To examine whether these differences in resistance arose from constitutive changes in defence responses, or from priming of inducible defences in leaves, we used quantitative RT-PCR to examine the expression of marker genes for JA- and SA-dependent defences in leaves. To activate JA-dependent defence, we used mechanical wounding of Col-0 and *OLE2::AOS* lines. In unwounded plants, expression of all three genes tested (*AOS*, *VSP2* and *PDF1.2*) was similar, indicating that the transgenic plants do not constitutively express wound-induced genes (Fig. 6c-e). Notably, the observation that expression of *AOS* in unwounded leaves did not differ between *OLE2::AOS* lines and the wild type control, confirms that *AOS* over-expression from the *OLE2* promoter is restricted to seeds. In wounded leaves, however, the levels of wound-induced expression was markedly higher in transgenic plants than in Col-0 for all three genes tested, suggesting primed responses to wounding as a consequence of JA accumulation in seeds. Conversely, we found that expression of the SA-dependent marker, *PR1*, was attenuated in transgenic lines compared to the response in wild-type Col-0 following *P. syringae* inoculation (Fig. 6f). *PR1* expression did not differ between mock-inoculated control and transgenic plants.

Discussion

We show here that maternal herbivory has a major impact on seed dormancy in Arabidopsis, a response which is mediated via JA-Ile signalling during seed development. The maternal environment is well-known to influence offspring phenotypes in both animals and plants (Roach & Wulff, 1987; Mousseau & Fox, 1988). Often, such effects are mediated by maternal provisioning to seed. For example, maternal herbivory has been shown to affect seed size and defensive metabolite content (Agrawal *et al*., 1999; Agrawal, 2001). In the case of seed dormancy, maternal experience of

day length and temperature function as seasonal cues to regulate the timing of offspring germination (Fenner, 1991; Donohue, 2009; Kendall & Penfield, 2012). Maternal experience of nitrate, water status and salinity has also been shown to influence germination behaviour (Fenner, 1991; He *et al*., 2014; Vu *et al*., 2015). Hence, maternal effects on seed dormancy in response to the abiotic environment are widespread and well-characterised. The data presented here, however, represent to our knowledge, the first characterisation of a change in seed dormancy in response to biotic stress.

The loss of dormancy caused by maternal herbivory appears to be mediated by jasmonate-dependent changes in amounts and/or sensitivity to ABA and GA. When levels of JA-Ile in dry seed are elevated, either as a consequence of parental herbivory or from transgenic over-expression of *AOS* during seed development, dormancy is significantly reduced, and at least in the case of simulated herbivory, this response is lost in JA signalling mutants. Elevated JA-Ile is associated with elevated gibberellin content and reduced ABA sensitivity in mature seed. This latter effect is consistent with earlier reports that the *jar1-1* and *coi1-16* mutants, which are impaired in JA signalling, are hypersensitive to ABA (Staswick *et al*., 1992; Ellis & Turner, 2002). The ABA hypersensitivity displayed by these mutants is also consistent with our observation of enhanced dormancy in *jar1-1* and *jin1-9*, which implies a role for JA-Ile in controlling dormancy during seed development in the absence of biotic stress. This idea is further supported by the correlation between seed JA content and degree of dormancy in the Col-0 and Cvi accessions of Arabidopsis, where seeds of the more dormant Cvi accession contain 10-fold lower levels of JA (Preston *et al*., 2009). Taken together, the germination phenotypes of seed from herbivore-infested plants, *OLE2::AOS* lines and the *jar1-1*, *coi1-16* and *jin1-9* mutants, demonstrate that JA-Ile plays an important role during seed development in regulating seed dormancy via effects on ABA and GA.

Interestingly, despite the fact that increased endogenous JA-Ile content promotes germination, exogenous application of JA to mature seeds inhibits germination, although only at high, non-physiological concentrations (Dave *et al*., 2011). OPDA is a more potent inhibitor of germination in Arabidopsis, and the elevated OPDA levels found in the *cts* mutant impose a strong block in germination, despite the fact that *cts* seed also contain elevated JA, JA-Ile and GA (Dave *et al*., 2011; 2016). Hence, in seed accumulating JA-Ile but not OPDA, such as those investigated in our study, the increase in GA levels and reduction in ABA sensitivity is sufficient to reduce dormancy, whilst in *cts* mutants, the ability of OPDA to inhibit germination via multiple dormancy-inducing pathways appears to be dominant over the effect of JA-Ile. The different roles of JA-Ile and OPDA also explain why Dave *et al*., (2011) saw no effect on dormancy in the JA-deficient *aos* mutant, whilst in the work

presented here, we report increased dormancy in *jar1-1* and *jin1-9*. These latter mutants still produce dormancy-promoting OPDA, but lack the dormancy-inhibitory response to JA-Ile, whereas the *aos* mutant is deficient in both OPDA and JA-Ile.

Progeny of mite-infested plants also displayed primed JA-dependent resistance responses to herbivores. This phenotype persisted across a stress-free generation into the SM_2, suggesting a transgenerational epigenetic effect, as was previously demonstrated in plants attacked by caterpillars or infected by *P. syringae* (Luna *et al*., 2012; Rasmann *et al*., 2012). In these cases, there is evidence of a role for the RNA-dependent DNA methylation (RdDM) pathway in establishing defence priming. Our experiments show that JA-Ile synthesised in the seed is sufficient to activate priming, and we have previously demonstrated that treatment of mature seed with JA is also able to prime herbivore resistance responses (Worrall *et al*., 2012). However, caterpillar herbivory for a limited period prior to flowering also primed defences in Arabidopsis without altering seed hormone profiles (Rasmann *et al*., 2012). Hence, maternal JA responses are sufficient to activate transgenerational defence priming without changes in seed hormones, and second generation plants maintain the priming phenotype without further exposure to elevated JA-Ile. In contrast, we detected effects of herbivory on seed dormancy only in the SM_1 generation, suggesting that this is a direct maternal effect that operates independently from defence priming and may require direct exposure of seeds to elevated JA-Ile. Whilst our experiments demonstrate that elevated seed JA-Ile is sufficient to generate both priming and dormancy effects, they do not distinguish whether the seed jasmonates accumulating during herbivory originate in the maternal plant, or are synthesized *de novo* by the zygote.

An important question which arises from the discovery of the effect of maternal herbivory on dormancy is whether this response has any adaptive significance for the plant. In the case of transgenerational defence priming, the assumption is that this response may have an adaptive benefit when the maternal environment is a good predictor of offspring environment (Holeski et al., 2012). When the parent plant is exposed to high pest or disease pressure, enhanced defence in the next generation would be selected for if offspring were similarly exposed to biotic stress. It is less clear what benefit might arise from precocious germination in the presence of herbivores, since that might increase the likelihood of seedling mortality. One possibility is that since primed plants would be better defended from herbivores, early germination may be mechanism to maximise this competitive advantage over non-primed individuals. Indeed, even in the absence of priming, early germination can increase fitness when selection acts on post-germination survival (Donohue *et al*., 2010). However, it must be recognised that our experiments were all conducted in controlled

environments. How other important environmental factors, such as temperature and day length, interact with herbivore stress to control dormancy in nature, has yet to be determined.

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Author Contributions

MRR conceived the study; MRR, IAG, FEV, SP and AD designed experiments; SP, AD, FEV, DW, GH, JW, FK and MRR performed experiments and analysed the data; MRR wrote the paper.

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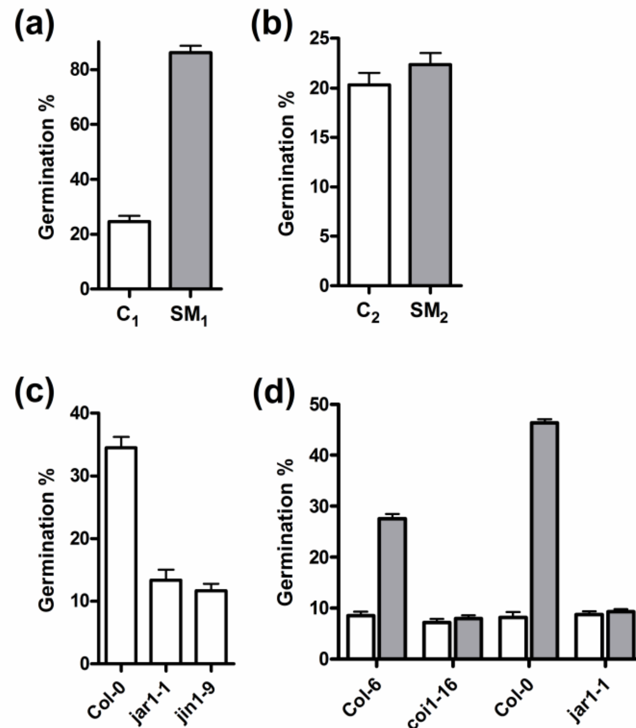

Figure 1. Primary dormancy in SM_1 and SM_2 seeds. Percentage germination seven days after sowing freshly-harvested Arabidopsis seed from first (**a**) and second (**b**) generation plants following maternal herbivory. C_1 and SM_1 populations represent first generation seed collected from control and stressed parents, whilst C_2 and SM_2 populations represent second generation seed collected from C_1 and SM_1 lines grown in stress-free conditions. (**c**) Dormancy of wild-type Col-0 and the *jar1-1* and *jin1-9* mutants. Data represent mean germination frequencies plus standard error from three replicates per experiment, with each replicate including 100-200 seed per plate. Data in (**a**) show combined results from three independent experiments, each experiment comprising three treatment replicates (n=9). Data were compared with a Mann-Whitney test ($p < 0.0001$). There was no significant difference between C_2 and SM_2 lines (Student's *t*-test; $P = 0.3$; n=3). Germination of *jar1-1* and *jin1-9* seed is significantly different from that in Col-0 ($P < 0.001$; 1-way ANOVA with Tukey post-test; n=3). (d) Effect of simulated herbivory on dormancy in JA mutants. Germination seven days after sowing freshly-harvested seed from the *coi1-16* and *jar1-1* mutants and their respective wild-type backgrounds either with, (grey bars) or without (white bars), maternal simulated herbivory. Data show mean percentage germination plus standard error from five individual plants of each genotype. Two-way ANOVA indicates a significant interaction between genotype and simulated herbivory ($P < 0.0001$).

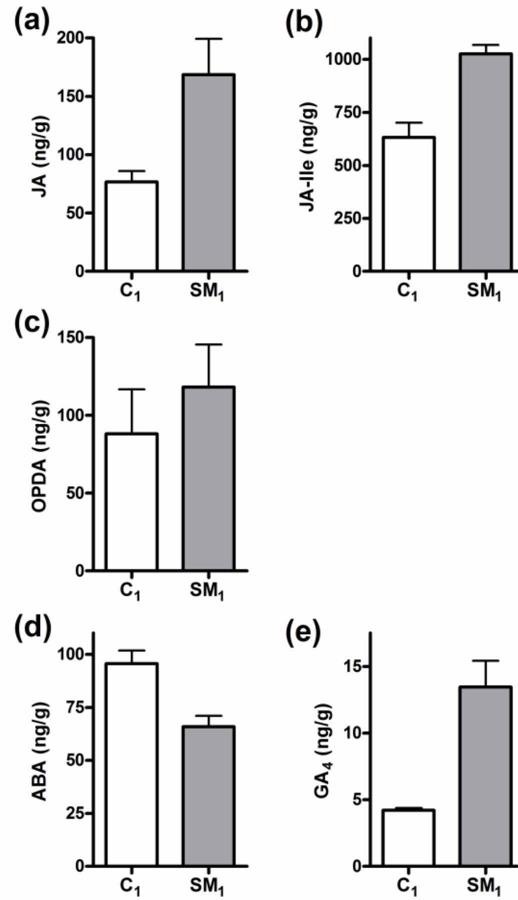

Figure 2. Hormone profiles in C_1 and SM_1 dry seed. Levels of the jasmonates jasmonic acid (**a**), jasmonoyl-L-isoleucine (**b**) and 12-oxo-phytodienoic acid (**c**), and of abscisic acid (**d**) and the biologically-active gibberellin, GA_4 (E), in C_1 and SM_1 Arabidopsis seed. Data represent means plus standard error of measurements from three independent C_1 and SM_1 lines. Student's *t*-test indicates that the quantities of JA ($P = 0.045$), JA-Ile ($P = 0.009$), ABA ($P = 0.02$) and GA_4 ($P = 0.009$) are significantly altered by maternal herbivory.

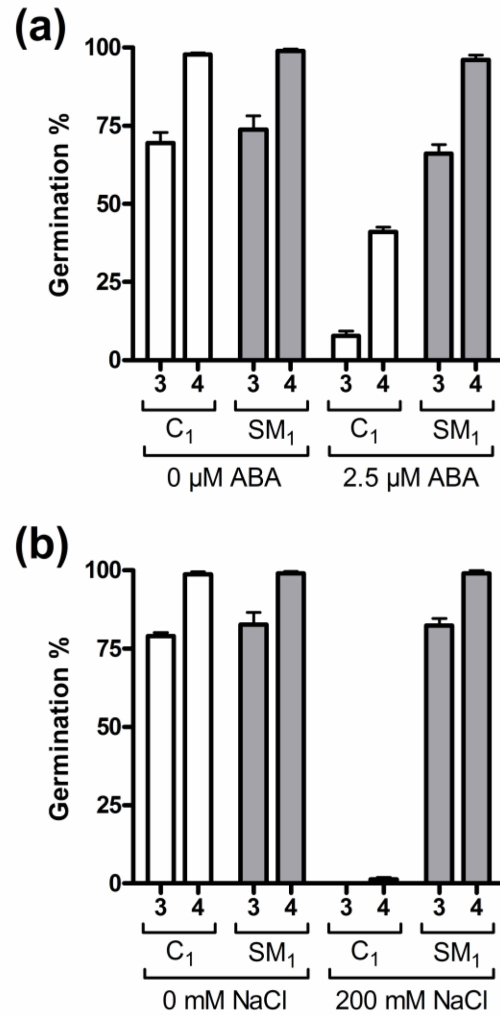

Figure 3. ABA sensitivity of germination in SM_1 Arabidopsis seed. Percentage germination three and four days after cold-stratification of seed from first generation plants on 2.5 µM ABA (**a**) and on 200 mM NaCl (**b**). Data represent means plus standard error of three replicate plates, where germination of 80-100 seed was scored on each plate. Open bars represent C_1 lines, whilst grey bars represent SM_1 lines. Numbers under each bar indicate days since stratification. Germination data from days 1-4 were analysed using a 2x2 factorial repeated-measures ANOVA general linear model in the IBM SPSS Statistics package. For the data sets in both panels, there was a significant interaction between time and seed line ($P < 0.001$), indicating that maternal herbivory affects germination.

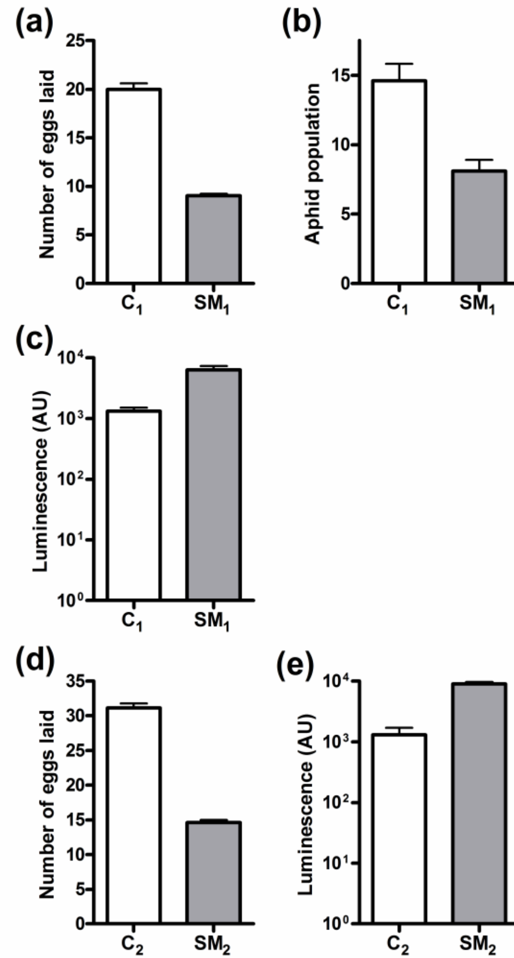

Figure 4. Offspring defence phenotypes following maternal herbivory.
Reproductive performance of red spider mite, *T. urticae* (**a, d**), populations of the aphid *M. persicae* (**b**), and bacterial growth (**c, e**) in three independent lines of Arabidopsis derived from parents of control or spider mite-infested plants. Bacterial growth was assessed by measuring luminescence (arbitrary units) in leaf extracts following inoculation with the bioluminescent strain *P. syringae* DC3000-*lux*. Plots in **a**, **b** & **d** show means plus standard error, plots in **c** and **e** show means plus standard deviation. Data were tested by Student's *t*-test. All SM_1 and SM_2 lines were significantly different from the respective C_1 and C_2 lines at $P < 0.0001$. All experiments included multiple individual plants from three independent C_1/C_2 and SM_1/SM_2 lines, and were conducted on 3 separate occasions: **a**; n = 63, **b**; n = 27, **c**; n = 18, **d**; n = 42, **e**; n = 36).

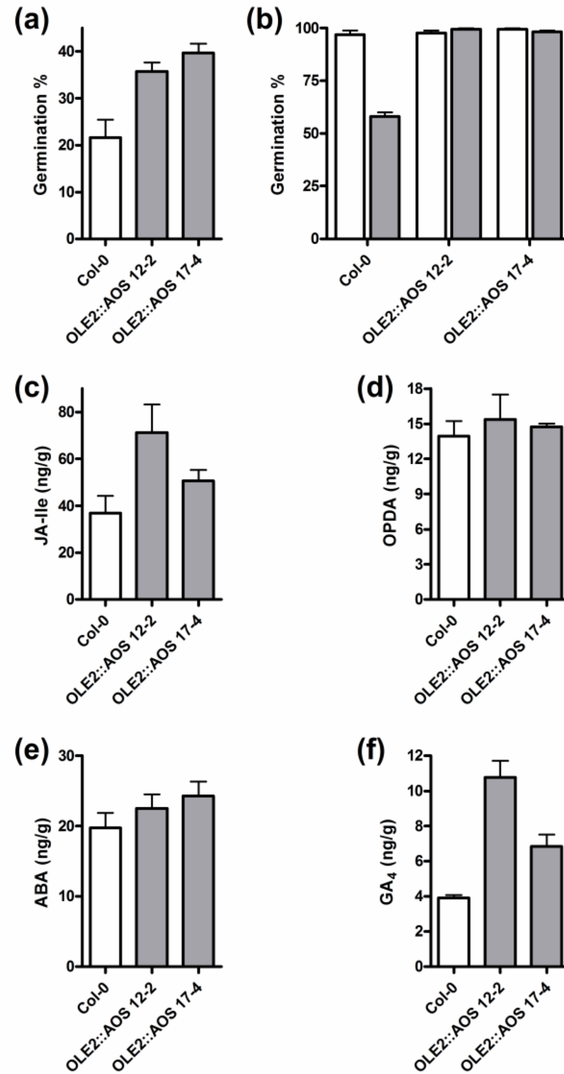

Figure 5. JA signalling reduces dormancy in Arabidopsis seeds. (**a**) Percentage germination seven days after sowing freshly-harvested seed from wild-type Col-0 and transgenic *OLE2::AOS* Arabidopsis lines. Data represent mean germination frequencies plus standard error from 3 replicates per line, with each replicate including 100 seed per plate. 1-way ANOVA indicates that germination is affected by the OLE2::AOS transgene (P = 0.008) (**b**) Percentage germination five days after sowing cold-stratified seed from wild-type Col-0 and transgenic *OLE2::AOS* Arabidopsis lines in the presence (grey bars) or absence (open bars) of 2.5 µM ABA. Data represent means plus standard error of three replicate plates, where germination of 80 seed was scored on each plate. (**c**-**f**) Hormone levels in *OLE2::AOS* lines. Data represent mean plus standard error of measurements from three independent plants from each line.

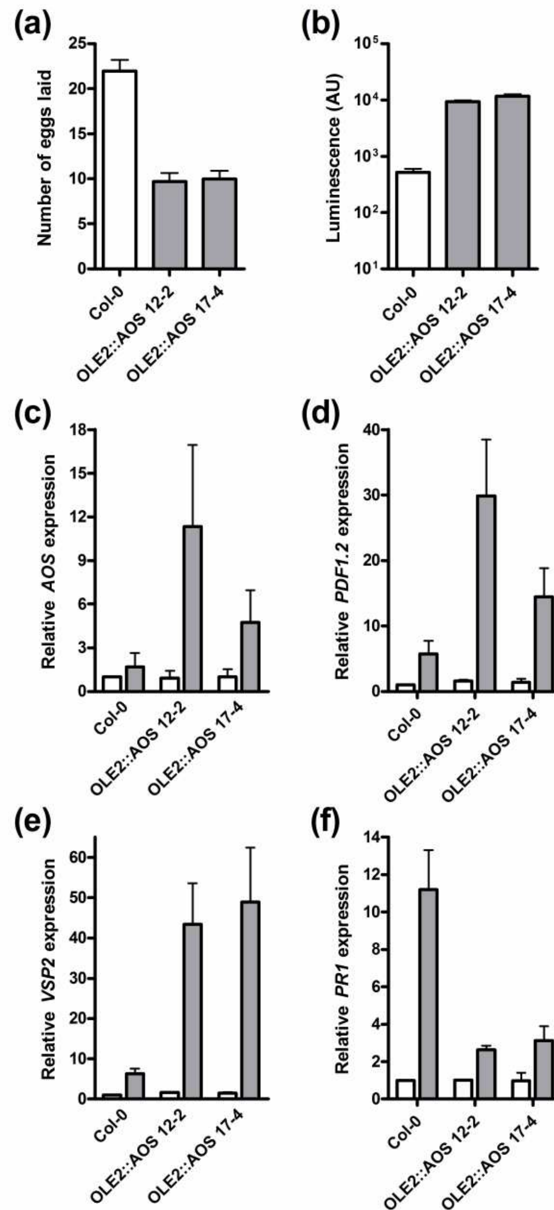

Figure 6. Defence phenotypes in Arabidopsis *OLE2::AOS* lines. (**a**) Reproductive performance of red spider mite, *T. urticae*, and (**b**) bacterial growth, (luminescence (arbitrary units) of leaf extracts) following inoculation with *P. syringae* DC3000-*lux* in wild-type Col-0 and transgenic *OLE2::AOS* lines. Plots show means plus standard error. 1-way ANOVA indicates that resistance to herbivory and disease are significantly different from Col-0 in both transgenic lines ($P < 0.0001$ (**a**; n = 21, **b**; n = 6)). (**c-f**) Defence-related gene expression in *OLE2::AOS* transgenic lines. Expression of the JA-regulated marker genes *AOS*; At5g42650 (**c**), *PDF1.2*; At5g44420 (**d**) and *VSP2*; At5g24770 (**e**) in control (white bars) or wounded leaves (grey bars), harvested 12 hours after initial wounding. (**f**) Expression of *PR1*; At2g14610 18 h following inoculation with *P. syringae* DC3000-*lux* in mock- (white bars) and bacterially-inoculated (grey bars) plants. Steady-state mRNA levels for each gene were determined

by qPCR and are expressed relative to an *ACT2*; At3g18780 internal control and normalised to the expression in Col-0 control plants. Error bars represent standard error of two independent biological replicates, in which each treatment group comprised eight individual plants.

Fig. S1 Simulated herbivory reduces primary seed dormancy in Col-0. Percentage germination 6 days after sowing freshly-harvested seed from plants subjected to simulated herbivory. NWC; non-wounded control, WBF; plants whose rosette leaves were wounded before flowering, WDF; plants whose rosette leaves were wounded during flowering. Bars show mean plus standard error for % germination for a minimum of 100 seed from each of several individual plants (NWC; n=14, WBF; n=9, WDF; n=10). ANOVA indicates a significant effect of wounding on dormancy ($P < 0.0001$).

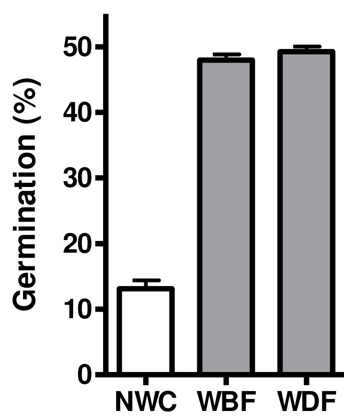

Fig. S2 Effects of ABA and NaCl on germination and seedling root growth in SM_1 and SM_2 lines. (**a**) Percentage germination two and three days after cold-stratification of seed of second generation plants on agar supplemented with 2.5 µM ABA. Numbers under each bar indicate days following stratification. Germination data from days 1-4 were analysed using a 2x2 factorial repeated-measures ANOVA general linear model in the IBM SPSS Statistics package. There was no interaction between time and seed line (P = 0.62), indicating that grandparental herbivory does not affect germination. (**b**) Length of the primary root in seedlings grown for seven days on vertical agar plates containing zero or 200 mM NaCl. Open bars represent C_1 and C_2 lines, whilst grey bars represent SM_1 and SM_2 lines. Data show means plus standard error from three independent lines. Data were analysed by 2-way ANOVA. C_1, but not SM_1 lines, show a significant decrease in primary root length under salinity (asterisk - interaction between line and salt treatment; P = 0.016). There was no significant effect of ABA treatment on germination in SM_2 lines.

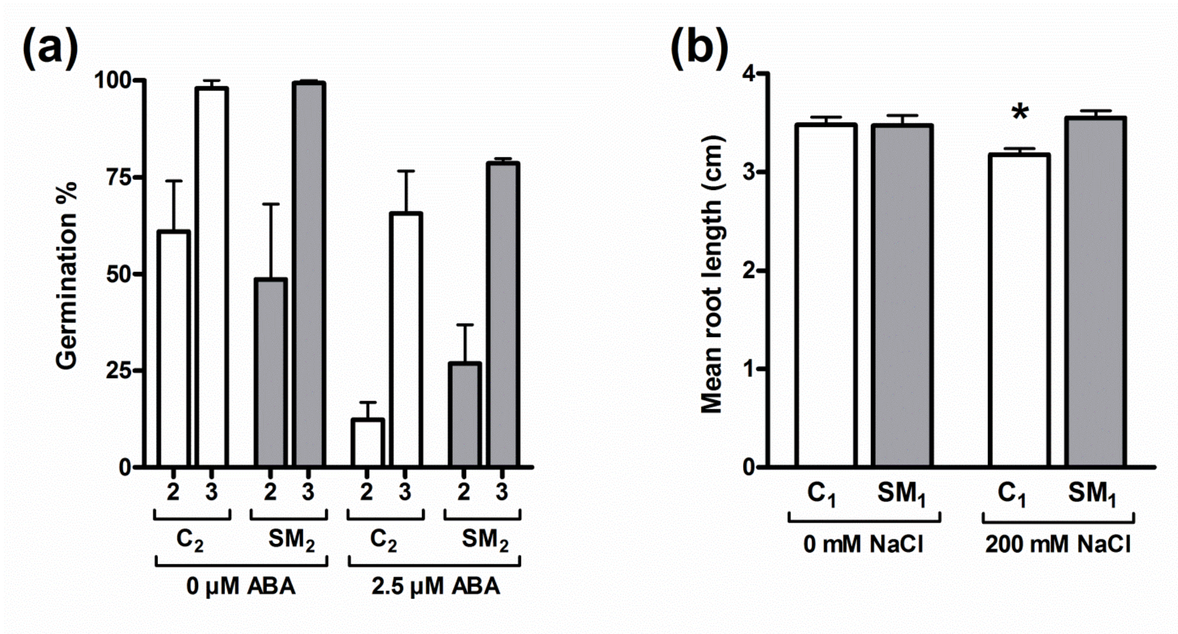

Fig. S3 *AOS* expression in seeds of the two transgenic lines selected for analysis. Steady-state mRNA levels were assessed by RT-qPCR using total RNA extracted from dry seeds. *AOS* expression was determined relative to an *ACT2* internal control and normalised to the expression in the Col-0 wild type background. Error bars represent standard deviation of 3 technical replicates.

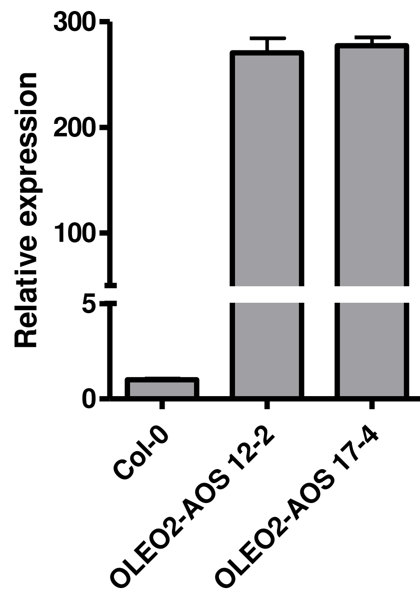

Table S1 Oligonucleotide primers used for cloning and qRT-PCR

Cloning	
Ole2-Pro-EcoRI-Fwd	CCCGAATTCGCAAAATAAAAGTGGTCGAAC
Ole2-Pro-NcoI-Rev	TTTCCATGGTGTAAGCTAATGAGAAATTGT
AOS-OE-NcoI-Fwd	TTTCCATGGATGGCTTCTATTTCAACCCCTTTT
AOS-OE-XmaI-Rev	TTTCCCGGGCTAAAAGCTAGCTTTCCTTAACGA
qPCR	
oligo dT	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTAT
PDF1.2-F	CGAGAAGCCAAGTGGGACAT
PDF1.2-F	TCCATGTTTGGCTCCTTCAA
VSP2-F	AATGTCACTCTCGACAATCTCGAA
VSP2-R	GGCTTCAATATGAGATGCTTCCA
PR-1-F	GTCTCCGCCGTGAACATGT
PR-1-R	CGTGTTCGCAGCGTAGTTGT
AOS-F	AAGTCAAAGCCGGTGAAAT
AOS-R	CTTACCGGCGCATTGTTTAT
ACT2-F	TGAGAGATTCAGATGCCCAGA
ACT2-R	TGGATTCCAGCAGCTTCCAT