EMSY-Like Genes Are Required for Full *RPP7*-Mediated Race-Specific Immunity and Basal Defense in *Arabidopsis*

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The *Arabidopsis thaliana* gene *enhanced downy mildew 2* (*EDM2*) encodes a nuclear protein required for *RPP7*-mediated race-specific disease resistance against *Hyaloperonospora arabidopsidis*, proper floral transition and additional developmental processes. Transcript levels of the disease-resistance gene *RPP7* are enhanced by *EDM2* while those of the floral suppressor *FLC* are repressed by *EDM2*. By yeast two-hybrid screening for *EDM2*-interacting proteins, we identified *AtEML1*, a member of a small group of four *Arabidopsis* proteins containing an EMSY N-terminal domain, a central Agenet domain, and a C-terminal coiled-coil motif. Using T-DNA mutants combined with silencing by artificial microRNAs, we found *AtEML1*, *AtEML2*, and, likely, *AtEML4* to contribute to *RPP7*-mediated immunity. Besides this, *AtEML1* and *AtEML2* participate in a second *EDM2*-dependent function and affect floral transition. Unlike *EDM2*, whose role in immunity appears to be limited to *RPP7*-mediated disease resistance, some *AtEML* members contribute to basal defense, an unspecific general defense mechanism. Domain architectures of *EDM2* as well as *AtEML* proteins suggest roles of these proteins in the regulation of chromatin states. Thus, possible cooperation of *AtEML* members with *EDM2* at the level of chromatin dynamics may link race-specific pathogen recognition to general defense mechanisms.

Two interconnected regulatory systems are known to mediate nonself recognition and innate immunity in plants (Dodds and Rathjen 2010). Pattern-triggered immunity (PTI) is based on specific recognition of broadly conserved molecular signatures of pathogens termed pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by plant pattern-recognition receptors (PRR) (Segonzac and Zipfel 2011). Effector molecules secreted by virulent pathogens can attenuate PTI, leading to a weakened immune response termed basal defense (Nimchuk et al. 2003). Recognition of effectors by plant disease resistance (R) proteins results in strong effector-triggered immunity (ETI), which efficiently prevents infections by avirulent biotrophic pathogens (Chisholm et al. 2006; Jones and Dangl 2006). ETI is race specific, because effectors and their complementary cognate R proteins are typically only present in pairs of corresponding pathogen/plant races and are not con-

served at the species level. A hallmark of ETI is the hypersensitive response (HR), the programmed death of plant cells at pathogen infection sites.

A complex regulatory network integrates distinct defense signals and coordinates various types of immune responses (Sato et al. 2010; Tsuda et al. 2009). Although both PTI and ETI involve a common set of downstream responses, such as transcriptional reprogramming of a core set of defense genes (Eulgem et al. 2004), production of reactive oxygen intermediates (ROI) (Torres et al. 2002), and the accumulation of salicylic acid (SA) and jasmonic acid (Glazebrook 2005), regulatory circuits connecting PAMP or MAMP and effector recognition are still largely unknown.

Several recent studies uncovered connections between floral transition pathways and innate immunity (Jin et al. 2008; March-Diaz et al. 2008; Tör et al. 2002; Tsuchiya and Eulgem 2010a; Wang et al. 2011). We reported that enhanced downy mildew 2 (*EDM2*), which is required for race-specific resistance of *Arabidopsis* against the Hiks1 isolate of the oomycete *Hyaloperonospora arabidopsidis*, also positively affects floral transition (Tsuchiya and Eulgem 2010a). Like mutants of the autonomous floral promotion pathway, mutants of *EDM2* show a photoperiod-independent late-flowering phenotype. Further analysis revealed that *EDM2* has a promoting effect on floral transition by suppressing transcript levels of the floral repressor gene *FLC*. *EDM2* appears to physically interact with the protein kinase WNK8, which acts in this pathway upstream from *EDM2* and counteracts *EDM2* function.

EDM2 is a nuclear protein containing an acidic N-terminal region, two PHD-finger-like domains, a Gy-like (GGL)-related protein–protein interaction domain we termed the plant GGL-related (PGR) domain, two putative bipartite nuclear localization signals, the ELP domain, which is a conserved sequence of unknown function, and a C-terminal proline-rich region (Eulgem et al. 2007; Tsuchiya and Eulgem 2010a). Physical interactions with WNK8 involve the PGR domain of *EDM2* and a region of the kinase containing a putative coiled-coil structure (Tsuchiya and Eulgem 2010a). Although WNK8 affects the roles of *EDM2* in flowering time control and other developmental processes (Tsuchiya and Eulgem 2010b), it seems not to have any detectable effect on *EDM2*-mediated immunity (Tsuchiya and Eulgem 2010a).

EDM2 enhances transcript levels of the *R* gene *RPP7*, which is required for race-specific immunity of *Arabidopsis* against *H. arabidopsidis* Hiks1 (Eulgem et al. 2007). The role of *EDM2* in immunity is highly specific. Whereas many other regulators required for *R* gene function have broader roles in immunity, *EDM2* appears only to affect disease resistance against *H.*

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arabidopsis Hiks1. It does not contribute to basal defense and no *R* genes, besides *RPP7*, have been reported to be dependent on EDM2. Mutations in *EDM2* phenocopy defense-related effects of *rpp7* mutants (Eulgem et al. 2007). In both types of mutants, *H. arabidopsis* Hiks1-induced HR and ROI production are abolished and expression of a common set of defense-related genes is affected. Taken together, these observations suggested that EDM2 acts at least in part through *RPP7*, by regulating transcript levels of this *R* gene.

Here, we report data functionally linking EDM2 to members of an *Arabidopsis* family of four EMSY-like (AtEML) proteins. AtEML proteins have possible roles in chromatin regulation and are related to the BRCA2-interacting human oncoprotein EMSY (Hughes-Davies et al. 2003). *AtEML1* and *AtEML2*, along with at least one additional member of this set of genes, have overlapping roles in activating *EDM2*- or *RPP7*-dependent immunity against *H. arabidopsis* Hiks1, while *AtEML1* and *AtEML2* appear to counteract the promoting effect of *EDM2* on floral transition. In contrast to *edm2* mutants, knock-down of *AtEML* members reduced basal defense against a virulent *H. arabidopsis* isolate. Thus, AtEML proteins have a broader role in plant immunity than EDM2. By interacting with EMSY-like proteins, EDM2 may bridge between an *H. arabidopsis* Hiks1-specific recognition mechanism and a more general defense function contributing to basal defense.

RESULTS

EDM2 can physically interact with AtEML1 in plant nuclei.

To identify EDM2-interacting proteins, we performed a yeast two-hybrid screen using the central portion of EDM2 (EDM2₂₁₄₋₁₂₀₄) fused to the GAL4 DNA-binding domain as a bait and several activation domain expression libraries representing pooled cDNAs from untreated and *H. arabidopsis*-infected Col-0 seedlings (Tsuchiya and Eulgem 2010a). Two independent, positive clones we identified encode the same protein containing an EMSY N-terminal (ENT) domain, a central Agenet domain, and a putative C-terminal coiled-coil structure. We termed this protein AtEMSY-like 1 (AtEML1; AT3G12140). The ENT domain of AtEML1 strongly resembles that of the human oncoprotein EMSY (Hughes-Davies et al. 2003). The *Arabidopsis* genome harbors three additional genes encoding proteins that are structurally closely related to AtEML1 and contain an N-terminal ENT domain, a central Agenet domain, and a C-terminal coiled-coil motif (Supplementary Fig. S1). We termed them *AtEML2* (AT5G06780), *AtEML3* (AT5G13020), and *AtEML4* (AT2G44440). We collectively refer to this set of four genes as *AtEML1/2/3/4*.

Our previous microarray analyses (Tsuchiya and Eulgem 2010b) revealed that constitutive transcript levels of all four *AtEML* genes are low (close to the noise range), with *AtEML4* transcript levels being the lowest. Relative to those of *AtEML4*, constitutive transcript levels of *AtEML1*, *AtEML2*, or *AtEML3* appear to be elevated by 2.6-, 3.7-, or 2.8-fold, respectively. Examining publicly accessible microarray data, we found transcripts of none of the *AtEML1/2/3/4* members to exhibit clear inducibility by any of a variety of biotic or abiotic stresses or hormone treatments. The only exception seems to be a moderate upregulation of *AtEML2* transcripts by heat stress.

Three splice forms are reported for AtEML1. AtEML1 version 1 (v1) and version 2 (v2) encode identical proteins. In the protein encoded by the third splice version (v3), the last 15 amino acids are replaced by a C-terminal extension comprised of 74 amino acids. By transiently expressing binary *CaMV35S*:GFP-AtEML1 expression constructs in *Nicotiana benthamiana* leaves, we found both AtEML1-v1 and AtEML1-v3 to localize to the nucleus, while green fluorescent

protein (GFP) alone also could be detected in the cell periphery (Fig. 1A).

To examine whether EDM2 and AtEML1 can physically interact in planta, we performed bimolecular fluorescence complementation (BiFC) assays (Citovsky et al. 2006) in tobacco leaves (Fig. 1B). Transient co-expression of the N-terminal half of yellow fluorescent protein (YFP) fused to the N terminus of the full-length EDM2 protein (nYFP-EDM2) and the C-terminal half of YFP fragment fused to full-length AtEML1-v1 (cYFP-AtEML1-v1) resulted in strong nuclear YFP fluorescence, indicating physical *in vivo* interactions between EDM2 and AtEML1 in nuclei. We obtained similar results with the closest AtEML1 paralog, AtEML2. No interactions were observed between nYFP-EDM2 and c-YFP-AtEML1-v3 or isolated cYFP (Fig. 1B).

AtEML1 and its *Arabidopsis* paralogs have overlapping roles in EDM2-mediated immunity.

Using a set of T-DNA mutants and transgenic *Arabidopsis* lines expressing artificial microRNAs (amiRNAs), we tested if *AtEML1/2/3/4* contribute to the immune function of EDM2. Because EDM2 and *RPP7* specifically mediate immunity of the *Arabidopsis* Col-0 and Col-5 accessions to *H. arabidopsis* Hiks1, all lines used in this study are of the Col-0 background (Table 1). We tested three independent mutants for *AtEML1* (*eml1-1*, *eml1-2*, and *eml1-3*), one *AtEML2* mutant (*eml2-1*), one *AtEML3* mutant (*eml3-1*), an *AtEML1/AtEML2* double mutant (*eml1-2/eml2-1*), and three independent lines expressing amiRNA co-silencing *AtEML3* and *AtEML4* transcripts in the *eml1-2/eml2-1* background (*eml-quad1*, *eml-quad2*, and *eml-quad3*). None of the tested single or double *eml* mutants or the *eml-quad* lines showed any obvious growth-related phenotype or developmental defects.

The intronic insertions of *eml1-1*, *eml1-2*, and *eml2-1* caused only a mild reduction of the transcript levels of *AtEML1* or *AtEML2*, respectively (Supplementary Fig. S2A and B). *AtEML1* transcripts are expressed at wild-type levels in the *eml1-3* mutant, which harbors an insertion in an exon of *AtEML1*. *AtEML3* transcript levels are strongly reduced (but still detectable) in *eml3-1* plants, which also contain an exonic insertion. Based on the nature of their insertion, only the *eml1-3* and *eml3-1* mutants are likely to constitute strong alleles, because the reading frames of the respective *AtEML* coding sequences are disrupted. The function of *AtEML* genes in *eml1-1*, *eml1-2*, or *eml2-1*, which contain intronic insertions, is likely to be only weakly compromised, because these mutants allow for near-wild-type levels of the respective transcripts. Only four lines of transgenic plants expressing the amiRNA co-silencing *AtEML3* and *AtEML4* in the *eml1-2/eml2-1* background could be recovered. Three of these *eml-quad* lines (*eml-quad1*, *eml-quad2*, and *eml-quad3*) contain single transgene insertions and were further characterized. In these lines, *AtEML3* and *AtEML4* transcript levels were reduced by only 30 to 60% compared with wild-type levels. The low number of transgenic lines we were able to recover and the partial nature of *AtEML3* and *AtEML4* silencing observed in them may indicate that full silencing of these genes is lethal.

In order to examine whether *AtEML1/2/3/4* contribute to the defense function of EDM2, we scored our set of *eml* mutants and amiRNA lines 7 days postinfection with *H. arabidopsis* Hiks1. Individual cotyledons of the infected plants were assigned to defined "bins" based on the strongest type of infection symptom they exhibited using the following classification scale: discrete HR sites (HR) < extended HR sites (eHR) < trailing necrosis (TN) < free *H. arabidopsis* hyphae (FH) < *H. arabidopsis* oospores (Oo) < *H. arabidopsis* sporangio-phores (Sp) (Supplementary Fig. S3). The category "HR" rep-

Fig. 1. *Enhanced downy mildew 2 (EDM2)* and *EMS-like 1 (AtEML1)* are co-localized and interact under in vivo conditions in *Nicotiana benthamiana* nuclei. **A**, Subcellular localization of green fluorescent protein (GFP) fused to two different AtEML1 splice forms, GFP-AtEML1 v1 and GFP-AtEML1 v3, in transiently transformed *N. benthamiana* leaf tissue. Fluorescence was observed by confocal microscopy. **B**, Bimolecular fluorescence complementation (BiFC) analysis of EDM2/AtEML1 and EDM2/AtEML2 interactions in vivo. BiFC constructs were transformed into *N. benthamiana* leaf epidermal cells by agroinfiltration. Four days postinoculation, yellow fluorescent protein (YFP) fluorescence was observed by confocal microscopy. YFP fluorescence results from complementation between the N-terminal (nYFP) and C-terminal (cYFP) parts of YFP fused to the N-termini of the respective tested proteins. Nuclear interactions with nYFP-EDM2 were only observed for cYFP-AtEML1-v1 and cYFP-AtEML2 but not cYFP-AtEML1-v3 or cYFP alone. **A** and **B**, Representative examples. 4',6-Diamidino-2-phenylindole (DAPI) staining was performed to specify locations of nuclei. Overlay: merged images of GFP or YFP, DAPI, and bright field. **B**, In any photographed tissue section, only a small number of fluorescing nuclei were in the plane of focus. However, we reproducibly observed for co-expression of nYFP-EDM2 with cYFP-AtEML1v1 or cYFP-AtEML2 a large number of BiFC-positive nuclei. .

resents a strong immune response not permitting the development of *H. arabidopsidis* hyphae, while “eHR” indicates that immunity is partially weakened, allowing limited growth of hyphae, which are still surrounded by plant cells undergoing HR cell death. In the case of trailing necrosis, HR trails extended *H. arabidopsidis* hyphae. Free hyphae are not surrounded or trailed by HR. Occasionally, the formation of sexual oospores is observed during successful *H. arabidopsidis* infections (Eulgem et al. 2007). Oospores as well as sporangiophores, which serve the release of asexual spores, represent

the successful conclusion of the *H. arabidopsidis* infection cycle (Holub and Beynon 1996).

None of the tested *eml* lines showed as strong a reduction of *H. arabidopsidis* Hiks1 resistance as *edm2-2* (Eulgem et al. 2007), which has nearly completely lost its immunity to this pathogen (Fig. 2A) (Tsuchiya and Eulgem 2010a). In Col-0 plants, there were no free *H. arabidopsidis* Hiks1 hyphae, oospores, or sporangiophores. We occasionally observed free hyphae in *eml1* single mutants. However, only the *eml1-2/eml2-1* double mutant and the *eml*-quad lines showed a significant in-

Table 1. Mutant and transgenic *Arabidopsis* lines used in this study

Name of line	AGI number for affected genes ^a	Comments
<i>eml</i> -like 1-1 (<i>eml1-1</i>)	AT3G12140	T-DNA insertion in intron of 5' untranslated region (UTR) (SALK_108872)
<i>eml</i> -like 1-2 (<i>eml1-2</i>)	AT3G12140	T-DNA insertion in fifth intron (SALK_114038)
<i>eml</i> -like 1-3 (<i>eml1-3</i>)	AT3G12140	T-DNA insertion on sixth exon (SALK_077088)
<i>eml</i> -like 2-1 (<i>eml2-1</i>)	AT5G06780	T-DNA insertion in intron of 5' UTR (SALK_116222)
<i>eml</i> -like 3-1 (<i>eml3-1</i>)	AT5G13020	T-DNA insertion in second exon (SALK_106147)
<i>eml1-2/eml2-1</i>	AT3G12140, AT5G06780	SALK_114038/ SALK_116222 double mutant
<i>eml</i> -quad-1	AT3G12140, AT5G06780, AT5G13020, AT2G44440	<i>EMSY-like3</i> (AT5G13020) and <i>EMSY-like4</i> (AT2G44440) silenced in <i>eml1-2/eml2-1</i> background
<i>eml</i> -quad-2	Same as <i>eml</i> -quad-1	Second independent <i>eml</i> -quad transgenic line
<i>eml</i> -quad-3	Same as <i>eml</i> -quad-1	Third independent <i>eml</i> -quad transgenic line

^a AGI = *Arabidopsis* Genome Initiative.

Fig. 2. Knock-down of *EMSY-like* (*AtEML*) genes affects enhanced downy mildew 2 (EDM2)-dependent immunity. **A**, Two-week-old *Arabidopsis* seedlings were spray infected with *Hyaloperonospora arabidopsidis* Hiks1 spores at 5×10^4 /ml and scored for disease symptoms 7 days later. Individual cotyledons of the infected plants were assigned to defined categories based on the strongest type of infection symptom they exhibited using the following classification scale: discrete hypersensitive response (HR) sites < extended HR sites (eHR) < trailing necrosis (TN) < free *H. arabidopsidis* hyphae (FH) < *H. arabidopsidis* oospores (Oo) < *H. arabidopsidis* sporangiophores (Sp) (see also Supplementary Figure S3). Based on the frequencies of eHR- and TN-containing cotyledons (*), χ^2 tests (Hartl and Jones 1998) showed highly significant differences ($P < 0.01$) between Col-0 on the one hand and *eml1-2/eml2-1* (*e1-1/e2-1*) as well as all three *eml*-quad (*eml-q*) lines on the other hand. Similarly, frequencies of eHR-, TN-, and FH-containing cotyledons differed highly significantly ($P < 0.01$) in *eml*-quad1 and *eml*-quad2 from those in the *eml1-2/eml2-1* line (**). **B**, *RPP7* transcript levels in 2-week-old seedlings of the indicated *Arabidopsis* lines determined by quantitative real-time reverse-transcription polymerase chain reaction. Data shown are based on averages from two biological replicates, each representing three technical replicates. Error bars represent standard deviations; * symbols mark significant changes ($P < 0.05$) relative to Col-0 as determined by Student's *t* tests, which were performed as described previously (Yuan et al. 2006).

crease of infection symptoms. The *eml1-2/eml2-1* double mutant exhibited a more than twofold higher frequency of eHR and TN cotyledons than Col-0. Because a significant reduction of *H. arabidopsidis* Hiks1 resistance was observed in the *eml1-2/eml2-1* double mutant but not in the *eml1-2* or *eml2-1* single mutants, both *AtEML1* and *AtEML2* play redundant roles in this defense mechanism.

Compared with *eml1-2/eml2-1* plants, the reduction of immunity was more pronounced in the *eml-quad* lines, which not only showed a clear increase in the number of eHR and TN cotyledons but also exhibited more cotyledons with free hyphae. Based on the occurrence of eHR-, TN-, and FH-containing cotyledons, the *eml-quad1* and *eml-quad2* lines were significantly more susceptible than the *eml1-2/eml2-1* double mutant. In addition, these two amiRNA lines occasionally allowed for the development of oospores and sporangiophores. Thus, either both *AtEML3* and *AtEML4* together or one of them alone enhances the immune function of *AtEML1* and *AtEML2*. Taken together, our results show that *AtEML1* and *AtEML2* along with one or both additional members of the *AtEML1/2/3/4* set act redundantly in promoting immunity against *H. arabidopsidis* Hiks1, which is also dependent on *EDM2*.

EDM2 is likely to contribute to *H. arabidopsidis* Hiks1 resistance by enhancing transcript levels of the *R* gene *RPP7* (Eulgem et al. 2007; Tsuchiya and Eulgem 2010a). Quantitative real-time reverse-transcription polymerase chain reaction (qPCR) showed *RPP7* transcript levels to be reduced by 40 to 50% in all tested *eml* and *eml-quad* lines, except for *eml3-1*. However, these changes were only significant for *eml1-2*, *eml1-2/eml2-1*, and two of the *eml-quad* lines (Fig. 2B). Consistent with the weaker nature of their *H. arabidopsidis* Hiks1 susceptibility phenotype, the reduction of *RPP7* transcript levels is less pronounced in the *eml1-2/eml2-1* mutant and *eml-quad* lines compared with *edm2-2* plants. Unlike all other tested *eml* mutants, *eml3-1* plants clearly did not show a reduction of *RPP7*

transcript levels. Thus, increased *H. arabidopsidis* Hiks1 susceptibility in the *eml-quad1* and *eml-quad2* lines compared with the *eml1-2/eml2-1* double mutant may reflect a role of *AtEML4* but not *AtEML3* in this *EDM2*-dependent defense pathway.

Although the *eml2-1* single mutant also exhibited a significant reduction of *RPP7* transcript levels, it appears not to be compromised in *H. arabidopsidis* Hiks1 resistance. This may indicate that *AtEML* genes may partially utilize defense mechanisms that are independent from *RPP7* and do not correlate with the levels of suppression of this *R* gene. The role of *AtEML* genes in basal defense described below supports such a scenario.

***AtEML* genes have a broader role in immunity than *EDM2*.**

The contribution of *EDM2* to immunity seems to be limited to the *RPP7* pathway, which specifically mediates strong resistance to *H. arabidopsidis* Hiks1. Neither basal defense to *H. arabidopsidis* nor race-specific resistance mediated by other characterized Col-0 *R* genes appears to be reduced in *edm2* mutants (Eulgem et al. 2007; Tsuchiya and Eulgem 2010a). To test whether the defense function of *AtEML* genes is also limited to resistance against *H. arabidopsidis* Hiks1, we infected *eml* mutants and *eml-quad* lines with the *H. arabidopsidis* isolate Noco2, which is virulent on Col-0 plants. As expected, the *edm2-2* mutant exhibited fully intact basal defense and behaved as susceptible to this *H. arabidopsidis* isolate as Col-0 plants (Fig. 3). Wild-type plants of the *Arabidopsis* Landsberg erecta (Ler) accession, which recognize *H. arabidopsidis* Noco2 by the *RPP5* *R* gene (Parker et al. 1993), were fully resistant. To our surprise, all three *eml-quad* lines exhibited significantly increased susceptibility to *H. arabidopsidis* Noco2 allowing for a two- to threefold or higher number of *H. arabidopsidis* spores to be produced than Col-0. However, compared with the massively enhanced susceptibility of the transgenic *nahG* line, which is known to be strongly compromised

Fig. 3. EMSY-like (*eml*) mutants and *eml-quad* lines are compromised in basal defense against *Hyaloperonospora arabidopsidis* Noco2. Three-week-old *Arabidopsis* seedlings of the indicated genotypes were spray-inoculated with the virulent *H. arabidopsidis* isolate Noco2 at 2×10^4 spores/ml. Numbers of spores per milligram fresh weight were counted at 7 days postinfection. Spore numbers are shown relative to those observed in Col-0 plants. Error bars represent standard deviations based on three independent biological replicates. Significant (*, $P < 0.05$) differences between average spore numbers were detected using Student's *t* tests.

in basal defense (Delaney et al. 1994), the reduction of basal defense in the *eml-quad* lines is moderate. Although our data do not allow to infer which AtEML1/2/3/4 members contribute to basal defense against *H. arabidopsidis* Noco2, clearly at least one representative of this set has a broader role in immunity than EDM2. The reduction of basal defense that we observed in *eml-quad* lines may reflect overlapping contributions of multiple AtEML1/2/3/4 members to this immune response, or a basal defense-related role of either AtEML3 or AtEML4.

AtEML genes negatively affect floral transition.

We previously found EDM2 to have a promoting effect on floral transition (Tsuchiya and Eulgem 2010a). To test whether AtEML1/2/3/4 also affect flowering time, we grew *eml* mutants and silencing lines under long-day conditions and counted the number of rosette leaves formed until bolting (Table 2). As observed previously, the *edm2-2* mutant flowered late compared with Col-0. The *eml1-3* mutant showed a moderate but significant early flowering phenotype, forming, on average, two to three fewer rosette leaves before bolting than Col-0 plants. The *eml2-1* mutant exhibited a similar trend, which is also significant but weaker. Floral transition was clearly accelerated in the *eml1-2/eml2-1* double mutant by three leaves compared with Col-0. This phenotype exceeded the weak effect observed in *eml2-1* plants. The flowering phenotype of *eml1-2/eml2-1* plants was not further enhanced in any of the *eml-quad* lines.

Table 2. AtEML1 and AtEML2 negatively affect floral transition^a

Line	No. of rosette leaves ^b	<i>t</i> test <i>P</i> values (versus Col-0)
Col-0	12.5 ± 0.2	...
<i>edm2-2</i>	17 ± 0.5	2.662E-08
<i>eml1-1</i>	12.6 ± 0.2	0.749
<i>eml1-2</i>	12.5 ± 0.3	0.947
<i>eml1-3</i>	9.7 ± 0.3	4.175E-07
<i>eml2-1</i>	11.4 ± 0.2	0.009
<i>eml1-2/eml2-1</i>	9.6 ± 0.2	4.654E-09
<i>eml-quad-1</i>	10.1 ± 0.3	1.806E-06
<i>eml-quad-2</i>	9.7 ± 0.2	2.269E-09
<i>eml-quad-3</i>	9.6 ± 0.3	3.102E-08

^a Flowering time during long-day conditions.
^b Number of rosette leaves formed until bolting. Total leaves: *n* = 20.

EDM2 promotes floral transition by suppressing transcript levels of the floral suppressor gene *FLC* (Tsuchiya and Eulgem 2010a). *FLC* transcript levels are enhanced in *edm2* mutants. Consistent with their early-flowering phenotype, we found, by qPCR, *FLC* transcript levels of *eml1-3*, *eml2-1*, *eml1-2/eml2-1*, and *eml-quad* plants to be significantly reduced compared with Col-0 and to accumulate to only approximately 50% of wild-type levels (Fig. 4). Although *FLC* transcript levels appear also to be reduced in *eml1-1* and *eml2-1* plants, these transcript changes were not significant. Our data indicate that AtEML1 and AtEML2 negatively affect floral transition and counteract the promoting effect of EDM2 on this developmental process. Similar to EDM2, the effect of AtEML1 and AtEML2 on floral transition is likely mediated by modulation of *FLC* transcript levels. These findings provide further support for a functional connection between AtEML genes and EDM2.

DISCUSSION

The *Arabidopsis* genome harbors nine genes encoding proteins with ENT domains (Hughes-Davies et al. 2003). We limited our study to a set of four closely related members of this family, which we termed AtEML1/2/3/4, and which encode proteins containing, besides the ENT domain, a central Agenet domain and a C-terminal coiled-coil motif. We found members of this gene set to affect plant innate immunity as well as floral transition. Compared with the strong defense-related phenotypes of the *edm2-2* or *nahG* lines that served as controls in this study, the reduction of *H. arabidopsidis* Hiks1 resistance or basal defense in *eml* mutant and silencing lines was only weak to moderate. Most likely, functional redundancy between individual members of the AtEML1/2/3/4 gene set as well as the use of weak alleles did not allow for a stronger expression of these phenotypes. For example, in the *eml-quad* lines, weak T-DNA alleles of AtEML1 and AtEML2 are combined with an amiRNA construct that only partially silences AtEML3 and AtEML4 transcripts. Nonetheless, the defense-related phenotypes we observed were very clear and reflect important contributions of the tested genes to the respective defense pathways.

AtEML1/2/3/4 have not been functionally characterized in *Arabidopsis*. The only plant gene of this type for which a functional study has been reported is *RIF1*. It encodes a nuclear protein of maize that interacts with the basic helix-loop-helix

Fig. 4. *FLC* transcript levels in 2-week-old seedlings of the indicated *Arabidopsis* genotypes grown under long-day conditions determined by quantitative real-time reverse-transcription polymerase chain reaction. Error bars represent standard deviations. Data shown are based on averages from two biological replicates, each representing three technical replicates. Error bars represent standard deviations; * symbols mark significant changes (*P* < 0.05) relative to Col-0 as determined by Student's *t* tests, which were performed as described previously (Yuan et al. 2006).

transcription factor R in the control of anthocyanin accumulation (Hernandez et al. 2007). The ENT domain was originally identified in the human oncoprotein EMSY, which appears to counteract the transcriptional activator function of the tumor suppressor BRCA2 (Hughes-Davies et al. 2003). The observation that EMSY also interacted with proteins involved in chromatin remodeling such as HP1 and BS69 (Hughes-Davies et al. 2003), as well as the fact that BRCA2 has histone acetyltransferase activity (Raouf et al. 2005; Siddique et al. 1998), linked EMSY to the regulation of chromatin dynamics. In addition to the N-terminal ENT domain, *AtEML1/2/3/4* harbor a central Agenet domain, which is related to epigenetic “reader” domains, such as the chromo domain or the tudor domain (Maurer-Stroh et al. 2003). Based on this, a general function of *AtEML1/2/3/4* as epigenetic chromatin-associated factors is highly likely.

Consistent with the ability of *AtEML1* and *AtEML2* to physically interact in *N. benthamiana* nuclei with the race-specific defense regulator EDM2, we found these two *AtEML* members and possibly at least one additional representative of this family to contribute to the immune function of EDM2. Functional links between EDM2 and *AtEML1/2/3/4* members are further supported by the involvement of *AtEML1* and *AtEML2* in flowering time control, which is a second process to which EDM2 contributes. Whereas EDM2 and *AtEML1/2/3/4* members promote resistance to *H. arabidopsidis* Hiks1 and *RPP7* expression, they have antagonistic functions in the control of floral transition and *FLC* expression. Our study did not address whether direct physical interactions between EDM2 and *AtEML1/2/3/4* proteins are relevant for any of these functions (defense and flowering). However, a hypothetical scenario explaining how two physically interacting proteins can cooperate in one process and antagonize each other in another process may be that the existence of a complex of both partners is sufficient for cooperation in the first case (e.g., defense), whereas counteracting activities of both partners in the same or different complexes account for antagonism in the second case (e.g., flowering).

The role of *AtEML1* and *AtEML2* in floral transition resembles that of the protein kinase gene *WNK8*, which also counteracts the promoting effect of EDM2 on this developmental step (Tsuchiya and Eulgem 2010a). In contrast to *AtEML1* and *AtEML2*, *WNK8*, however, appears not to affect the immune function of EDM2. Nevertheless, in *Arabidopsis*, a growing body of evidence hints at regulatory links between defense induction and floral transition. In addition to EDM2 and *AtEML* genes, several other defense components, including SA, have been found to affect flowering time control (Jin et al. 2008; March-Diaz et al. 2008; Tör et al. 2002; Wang et al. 2011). Therefore, functional links between these seemingly unrelated processes are unlikely to be coincidental. Pathogens with biotrophic or hemibiotrophic lifestyles are known to manipulate host development for their own benefit (Kay et al. 2007; Kazan and Manners 2009; Uchida and Tasaka 2010). In some cases, plants may take advantage of pathogen interference with host developmental programs and use such interference as a trigger for defense activation. Numerous studies have reported on regulatory circuits between pathogen-manipulated host processes and defense induction (Dodds and Rathjen 2010; Jones and Dangl 2006; Romer et al. 2007). Alternatively, plants may benefit from the modulation of their own developmental programs during pathogen infections and may “intentionally” accelerate or delay certain stages of their life cycle (Korves and Bergelson 2003). This may enable them to balance the allocation of energy and metabolic resources to immune responses with the need to properly maintain homeostasis of their own cells and tissues. It may even be an advantage for infected plants

to speed up the transition to reproduction and seed production in order to ensure reproductive success during disease. Scenarios in which plants actively change their own development in response to pathogen infections are supported by observations that abiotic stresses can have similar effects (Balasubramanian et al. 2006; Franks 2011; Kolar and Senkova 2008). Focused studies on regulatory connections between plant immune responses and developmental programs may reveal many interesting surprises in the future.

Intriguingly, members of the *AtEML1/2/3/4* gene set appear to have a wider role in immunity than EDM2, whose defense-related role seems to be limited to race-specific resistance against *H. arabidopsidis* Hiks1. Like the proteins encoded by *AtEML1/2/3/4*, primary structural features of EDM2, such as putative PHD-finger like epigenetic reader modules (Eulgem et al. 2007), point to a role of this protein in chromatin-associated transcriptional regulation. Thus, possible cooperation of *AtEML1/2/3/4* and EDM2 at the level of chromatin dynamics may link race-specific pathogen recognition to general defense mechanisms. A broad role of *AtEML1/2/3/4* in defense induction is consistent with the fact that such a function has also been demonstrated for BRCA2a, the *Arabidopsis* ortholog of the human EMSY-interactor BRCA2. BRCA2a was recently shown to be required for basal defense and to have a broad role in defense gene induction (Wang et al. 2010). It remains to be shown, however, to what extent details of the interactions between EMSY-like proteins and BRCA2 homologs and their regulatory roles are conserved between animal and plant systems.

Although the involvement of chromatin-associated regulation in flowering time control has been extensively examined (Dennis and Peacock 2007; He et al. 2003), the connection between these types of epigenetic processes and plant immunity is new and, thus far, only marginally explored. Only a small number of publications thus far have touched on this promising nascent area (Jaskiewicz et al. 2011; Palma et al. 2010; van den Burg and Takken 2009). With *AtEML1/2/3/4*, we have described here four interesting new components of the plant immune system that are likely to have roles in the regulation of chromatin states. In the case of floral transition, post-translational modifications of nucleosome core histones provide an epigenetic memory of expanded vernalization, which conditions some *Arabidopsis* accessions to flower after subsequent exposure to long-day conditions. Similarly, chromatin modifications have recently been shown to prime some defense genes after an initial defense-related stimulus to respond more intensely to secondary defense induction (Jaskiewicz et al. 2011). Whether or not *AtEML1/2/3/4* serve epigenetic memory processes in the control of flowering and defense induction will be an interesting subject of future studies.

MATERIALS AND METHODS

Yeast two-hybrid screens.

Yeast two-hybrid screens were performed as described previously (Tsuchiya and Eulgem 2010a).

BiFC assays and subcellular localization of GFP-fusion proteins.

BiFC assays were performed as described previously (Tsuchiya and Eulgem 2010a). Briefly, each cDNA of full-length EDM2, *AtEML1-v1*, or *AtEML-v3* was amplified by PCR and cloned into BiFC Gateway vectors (Citovsky et al. 2006) to examine in planta interactions between EDM2 and *AtEML-v1* or *AtEML-v3*. Expression cassettes to produce nEYFP₁₋₁₇₄-EDM2 and cEYFP_{175-end}-*AtEML-v1* or cEYFP_{175-end}-*AtEML-v3* were transferred into the I-*SceI* or I-*CeuI* sites of

the pPZP-RCS1 binary vector (Goderis et al. 2002). *Agrobacterium tumefaciens* GV3101 harboring the resulting BiFC binary vectors and a p19 silencing suppressor construct were co-infiltrated into the abaxial surface of 2- to 3-week-old *N. benthamiana* leaves as described previously (Goodin et al. 2002). YFP fluorescence was visualized in epidermal cell layers after 2 to 4 days of infiltration, using a Leica SP2 confocal microscope. Leaves were also infiltrated with 4',6-diamidino-2-phenylindole at 15 µg/ml for nuclei staining. Open reading frames of *AtEML1-v1* or *AtEML-v3* are also introduced into the pMDC43 vector (Curtis and Grossniklaus 2003) to produce GFP-*AtEML1-v1* or GFP-*AtEML1-v3*. GFP fluorescence was observed as described above.

Mutant lines.

Wild-type plants of the *Arabidopsis* accession Col-0 were obtained from the *Arabidopsis* Biological Resource Center (ABRC; Ohio State University, Columbus, U.S.A.). All experiments were performed exclusively in the Col-0 background. We obtained all used T-DNA mutants (Table 1) from the SALK T-DNA insertion collection from the ABRC (Alonso et al. 2003). The *edm2-2* allele has been described previously (Eulgem et al. 2007). The T-DNA insertion sites were confirmed by PCR-based genotyping using T-DNA border primers and gene-specific primers (Supplementary Table S1). Sequencing of T-DNA borders revealed errors in positions of T-DNA insertions at The Arabidopsis Information Resource database, such that the T-DNA insertion in *eml1-1* was found in the first intron while it was annotated in the second exon. The T-DNA insertion positions determined by sequencing are represented in Supplementary Figure S4. The *eml1/eml2* double mutant was produced by crossing *eml2-1* to *eml1-2* and genotyping their F2 progeny by PCR amplification.

Transgenic lines.

An amiRNA specifically targeting both *AtEML3* and *AtEML4* was designed using the Web MicroRNA Designer online tool against sequences conserved between these two genes (AT5G13020 and AT2G44440). To engineer the amiRNA, a total of six primers (A, B, I, II, III, and IV) were employed to initially perform three separate PCR assays using pRS300 (Schwab et al. 2006) as the template. A final overlap-PCR reaction using the flanking primer pair (A and B) was performed on the three primary PCR products as the template. To facilitate cloning into the Gateway system, we introduced *attB1* and *attB2* sites into primer A and B, respectively. The final PCR product was cloned into pDONR/Zeo (Invitrogen, Carlsbad, CA, U.S.A.). After sequence confirmation, the insert was recombined into the Gateway destination vector pEarleyGate100 (Earley et al. 2006) through LR-clonase (Invitrogen). pEarleyGate-EML was mobilized into the *A. tumefaciens* GV3101 and was used to transform the *eml1/eml2* double mutant by the floral dipping method (Clough and Bent 1998). The transformants were identified through BASTA selection. Transgenic *nahG* plants expressing an SA hydroxylase have been described previously (Delaney et al. 1994).

RNA isolation and reverse-transcription PCR analysis.

Total RNA was isolated from 2-week-old seedlings using Trizol reagent (Invitrogen). Total RNA (1 µg) was incubated with 1 unit of DNase I, RNase-free (Fermentas, Glen Burnie, MD, U.S.A.) in a total volume of 10 µl at 37°C for 30 min. After inactivation of DNase I by adding 1 µl of 50 mM EDTA and heating to 65°C for 10 min, RNA was reverse transcribed using 200 units of Maxima reverse transcriptase (Fermentas) and 100 pmol of oligo(dT)₁₈ primers in a sample volume of 20 µl. cDNA aliquots (0.5 µl) were used as template for real-time

qPCR using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). Each 25 µl of reaction mix contained 12.5 µl of Maxima SYBR green/fluorescein qPCR master mix (Fermentas) and 0.6 µM gene-specific primers. Agarose gel electrophoresis and thermal denaturation (melt curve analysis) were used to confirm specific replicon formation. For each reaction, the threshold cycle value (Ct) was determined by setting the threshold within the logarithmic amplification phase. Serial dilutions of total cDNA were used to calculate the amplification efficiency of each primer set according to the equation $E = 10^{-1/\text{slope}}$. Relative gene expression data were generated by the comparative Ct method using *Actin8* as a reference gene (Pfaffl 2001). All of the quantification experiments were done with at least two biological replicates in triplicate. All the primers were validated, and only those with efficiencies between 90 and 100% were used.

H. arabidopsidis isolates and infections.

The *H. arabidopsidis* isolates Hiks1 and Noco2 were described previously (Holub et al. 1994; Parker et al. 1993). *H. arabidopsidis* was grown, propagated, and applied to *Arabidopsis* as described previously (McDowell et al. 2000). Using Preval sprayers (Preval, Coal City, IL, U.S.A.), 1- or 3-week-old seedlings were spray inoculated with spore suspensions of *H. arabidopsidis* Hiks1 (5×10^4 spores/ml) or *H. arabidopsidis* Noco2 (2×10^4 spores/ml), respectively. Plants were scored at 7 days after infection for severity of infection by lactophenol trypan blue staining (Koch and Slusarenko 1990) or counting spores per plant fresh weight using a hemocytometer.

Flowering time measurement

Arabidopsis plants were grown at 22°C under long-day conditions (16 h of light and 8 h of darkness). Flowering time was measured as the number of rosette leaves formed at bolting.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Bio-Array Resource server: bar.utoronto.ca/welcome.htm
 The Arabidopsis Information Resource (TAIR) database:
www.arabidopsis.org
 Web MicroRNA Designer online tool: wmd.weigelworld.org