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Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in *Arabidopsis*

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Once attacked by herbivores, plants regenerate new leaves with increased trichome density as an inducible defense. Trichomes are specified from neighboring epidermal cells through local cell-cell interactions in the leaf primordia. However, the molecular mechanism of how herbivore-induced damage at older leaves remodels the pattern of trichome fate specification at newly forming leaves is largely unknown. In this study, we show that mutations in either the biosynthetic or signaling pathway of jasmonates (JAs), long-distance wound signals, abolish the wound-induced formation of trichomes. To identify the factors linking JA signaling to trichome fate specification, we isolated a novel class of mutants, *unarmed* (*urm*), which lack trichome induction but show otherwise normal responses to JAs. *URM9* encodes an Importin β family protein, and *URM23* is identical to *TRANSPARENT TESTA GLABRA1* (*TTG1*), the product of which interacts with the bHLH transcription factor GLABRA3 (GL3). Loss of either URM9 or URM23 disrupts the subnuclear localization of GL3, thus implicating GL3 in trichome induction. The expression of *GL3* was enhanced by JA treatment prior to trichome initiation. Genetic analysis of multiple trichome mutants shows that GL3, in concert with the R2R3-Myb transcription factor GLABRA1 (GL1), promotes trichome fate in response to JA in a dosage-dependent manner. These results indicate that GL3 is a key transcription factor of wound-induced trichome formation acting downstream of JA signaling in *Arabidopsis*.

KEY WORDS: Pattern formation, Cell fate, Plasticity, Trichome, Inducible defense, Jasmonate, Importin β, *Arabidopsis thaliana*, GLABRA3

INTRODUCTION

Environmental control of development (developmental plasticity) has advantageous adaptive roles in organisms that encounter variable environments. Various aquatic animals can sense chemical substances emitted by their predators and this induces morphological changes (e.g. the formation of thorns) to avoid predation (Tollrian and Harvell, 1998). Analogously, many plant species respond to herbivore-induced damage by regenerating new leaves with increased trichomes, which act as a physical barrier, providing defense against herbivores (Myers and Bazely, 1991). Trichome is a generic name for a myriad of hair-like epidermal cells (which may be unicellular or multicellular, glandular or non-glandular) in diverse plants, and there is some evidence that different types of trichomes are formed by different developmental programs, suggesting that trichomes have multiple evolutionary origins (Payne et al., 1999; Glover and Martin, 2000; Serna and Martin, 2006). We aimed to determine the molecular basis of wound-induced trichome formation using a model crucifer, *Arabidopsis thaliana* (L.) Heynh., with a view to future cross-species comparisons that might provide insight into the evolutionary origin of this inducible defense system.

The epidermis of *Arabidopsis* has been extensively studied as a model of cell-fate specification in plants. The immature epidermis of a leaf primordium is initially composed of uniform protodermal cells. Then, cell-cell interactions take place that specify three types of cell fate: pavement cells, trichomes and stomatal guard cells (Larkin et al., 2003). In *Arabidopsis*, a ternary complex composed of R2R3-Myb transcription factors [GLABRA1 (GL1; previously known as GLABROUS1) (Oppenheimer et al., 1991) or MYB23 (Kirik et al., 2005)], bHLH transcription factors [GLABRA3 (GL3) (Payne et al., 2000) or ENHANCER OF GLABRA3 (EGL3) (Zhang et al., 2003)] and the WD40 repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Walker et al., 1999) acts at the top of the regulatory hierarchy of trichome development. The Myb-bHLH-WD40 complex activates the expression of multiple downstream factors, each of which directs the morphogenesis or lateral inhibition of the developing trichomes (Szymanski et al., 1998; Schellmann et al., 2002; Ishida et al., 2007; Zhao et al., 2008). Thus, the current model of trichome patterning comprises a local autonomous circuit of multiple transcription factors acting at leaf primordia (Pesch and Hülskamp, 2004; Bouyer et al., 2008). However, herbivore-induced damage at older leaves does affect newly forming leaves and changes the frequency at which epidermal cells adopt the trichome fate, implying that a mobile signal must exist to control trichome fate specification in a systemic manner.

Jasmonic acid and derivative compounds, collectively referred to as jasmonates (JAs), act as the key signaling molecules that coordinate an array of diverse wound responses in plants. Wounded tissue rapidly activates JA biosynthesis (Glauser et al., 2008), and increased JA triggers the SKP1/Cullin/F-box E3 ubiquitin ligase complex containing the F-box subunit CORONATINE INSENSITIVE1 (SCF^{COI1}) to degrade the repressors of JA signaling – the JASMONATE-ZIM (JAZ) family proteins – by the ubiquitin/26S-proteasome pathway (Chini et al., 2007; Thines et al., 2007). In addition to their local synthesis and action, JAs also move systemically via vascular strands to transmit wound signals to distal tissues (Li et al., 2002; Thorpe et al., 2007). Therefore, JAs can be considered to be good candidates for the mediator of wound-induced trichome formation, which is also a systemic response. In support of this idea, exogenous JA treatment increases leaf trichome density in *Arabidopsis* and in tomato (*Solanum lycopersicum* L.) (Traw and Bergelson, 2003; Boughton et al., 2005). However, the significance

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and action of endogenous JAs during trichome development remain unknown. In this study, we demonstrate that endogenous JAs regulate trichome density by acting upstream of the Myb-bHLH-WD40 complex, and propose that JA-mediated control of *GL3* expression is the molecular basis linking wound response to trichome development in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* accession Columbia-0 (Col-0) was used as wild type. The following mutants and transgenic lines have been described previously: *aos* (Park et al., 2002); *coi1-1* (Xie et al., 1998); *egl3-7* (SALK_077439), *gl1-20990* and *gl3-sst* (Esch et al., 2003); *gl1-1* (Oppenheimer et al., 1991); *gl1-2* (Esch et al., 1994); *jar1-1* (Staswick et al., 2002); *myb23-1* (Kirik et al., 2005); *myc2-1* (Boter et al., 2004); *sad2-2* (Verslues et al., 2006); *GL1::GUS* (Larkin et al., 1993); *GL2::GUS* (Szymanski et al., 1998); *MYB23::GUS* (Kirik et al., 2001); *CPC::GFP* (Wada et al., 2002); *VSP1::GUS* (Ellis et al., 2001). *aos* and *coi1-1* were backcrossed to wild type Col-0 because the original lines had *gl1* markers and lacked trichomes. *gl3-11* (SALK_118201), *at2g31480-1* (SAIL_788_F09) and *at2g31480-2* (SAIL_857_D05) were identified from SALK and Syngenta (SAIL) T-DNA populations, respectively. *gl1-S92F* is identical to the Col-6 accession (ABRC stock CS8155). *gl2-3125* (ABRC stock CS3125) has a 1 bp deletion in the sixth exon of *GL2*. *ttg1-213* is a nonsense allele of *TTG1* (Trp183 to stop) that was isolated in our laboratory. Sequences of primers and details of restriction enzymes used for genotyping are available upon request.

Seeds were surface-sterilized and sown on 0.5% gellan-gum plates supplemented with 1 Murashige and Skoog salts without vitamins, with 1.5% sucrose, and adjusted to pH 5.8 with MES-KOH buffer. Seeded plates were kept at 4°C for 2 days and then incubated at 22°C under constant light. The second day at 22°C was defined as the day of germination [0 days after germination (DAG)]. For mechanical wounding, a pair of cotyledons and the first and second true leaves of each 8-DAG seedling were crushed with forceps spanning their midveins. For jasmonate treatment, a small strip of filter paper was placed in the center of a 6-DAG plate and methyl jasmonate (MeJA, a bioactive jasmonate) was absorbed onto the filter paper and allowed to vaporize (for details, see Fig. S1 in the supplementary material).

Microscopy

It is difficult to accurately compare the density of trichomes (trichome number per unit leaf area) because leaves continue to expand after the termination of trichome development (Larkin et al., 1996) and, moreover, the final leaf size is heavily influenced by the microenvironment. Instead, we used the trichome number per leaf as a more consistent index of trichome production. Total trichome number was counted by examining the adaxial side of the fifth true leaf under a stereomicroscope. For cryoscanning electron microscopy, fresh plant material was frozen in liquid nitrogen and observed under a FEI XL30 ESEM. For epifluorescence microscopy, a Leica MZ FLIII microscope equipped with a GFP2/3 filter and DC500 camera was used. For confocal microscopy, young leaf primordia were excised and mounted on a slide with water and observed under a Zeiss LSM510-META laser-scanning microscope.

Histochemical analysis

For histochemical GUS staining, 10-DAG seedlings (96 hours post MeJA treatment) were harvested and immersed in fixation/staining solution containing 1 mM X-Gluc, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 10 mM EDTA, 20% methanol and 0.3% Triton X-100 in 100 mM sodium phosphate buffer (pH 7.2). The samples were vacuum-infiltrated for 15 minutes at room temperature, incubated overnight at 37°C, then cleared in 70% ethanol.

Mutant screening

urm mutants were generated by EMS mutagenesis of *gl1-2* plants. Bulked M2 seeds were sown on soil and young seedlings sprayed with 2 mM MeJA. Plants devoid of trichome induction were isolated as candidate mutant lines. Screening of 11,000 M2 plants retrieved four *coi1* alleles among putative *urm* mutants, supporting the efficacy of our screening strategy. In this study, *urm9* and *urm23* were selected for further characterization.

Map-based cloning of *URM9* and *URM23*

urm9 gl1-2 and *urm23 gl1-2* were crossed to L*er gl1-2* BC10 (*gl1-2* was backcrossed ten times to the L*er* accession) to generate the mapping F2 populations. Approximately 80 and 900 F2 plants were used to pinpoint the *URM23* and *URM9* loci, respectively. New CAPS and SSLP markers were designed using information from the Monsanto *Arabidopsis* Polymorphism and L*er* Sequence Collection (http://www.arabidopsis.org/Cereon/index.jsp). Sequences of primers and details of restriction enzymes used for fine mapping are available upon request.

Molecular cloning and generation of transgenic plants

For transgenic complementation of *At2g31480*, a 4.1 kb genomic fragment containing the full-length *At2g31480* gene was PCR amplified and subcloned into a modified pPZP211 binary vector. For transgenic complementation of *At2g31660*, the *Arabidopsis* genomic BAC clone T9H9 was digested with *Eco*RI and *Spe*I (Verslues et al., 2006) and the longest (9.3 kb) fragment containing the full-length *At2g31660* gene was subcloned into a modified pPZP211 binary vector. For the construction of the *GL3::GL3-2xGFP* fusion, a 6.7 kb genomic fragment of *GL3*, including 3.2 kb upstream of the start with a deleted stop codon, was PCR-amplified, fused in frame to two tandem *GFP*s, then subcloned into a modified pPZP212 binary vector. Further details of construction are available upon request. Constructs were verified by sequencing and transformed into *Arabidopsis* plants (Col-0 and *urm9 gl1-2*) using the standard vacuum-infiltration method.

RESULTS

Endogenous JA regulates trichome patterning

In *Arabidopsis*, mechanical wounding to older leaves induces the formation of new leaves with increased trichome density (Fig. 1A,B,E) (Traw and Bergelson, 2003). Exogenous treatment of unwounded plants with jasmonic acid or methyl jasmonate (MeJA) mimics the effect of wounding and induces increased trichomes, suggesting the involvement of JA signal in trichome patterning events (Fig. 1C,D) (Traw and Bergelson, 2003). However, the role of endogenous JA remains unclear because *jasmonate resistant1-1* (*jar1-1*), a weakly JA-resistant mutant, shows normal induction of trichomes in response to wounding (Traw and Bergelson, 2003). To unequivocally address this issue, we used mutants completely lacking endogenous JA function. The *aos* mutant is deficient in JA biosynthesis owing to disruption of the *ALLENE OXIDE SYNTHASE* (*AOS*) gene, which encodes the key enzyme of JA biosynthesis (Park et al., 2002). Mechanical wounding of *aos* mutants did not increase trichome formation (Fig. 1E), although MeJA treatment of *aos* rescued the trichome density defects (data not shown). These data show that JA biosynthesis (catalyzed by AOS) is necessary and sufficient for trichome induction. *COI1* encodes an F-box subunit of the SCF E3 ubiquitin ligase complex (SCF^{COI1}) and is an essential component of the JA receptor (Xie et al., 1998; Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008). Like *aos*, *coi1-1* did not show increased trichomes in response to wounding (Fig. 1E), nor in response to MeJA treatment, indicating that canonical SCF^{COI1}-dependent JA signaling is responsible for trichome induction.

Interestingly, *aos* and *coi1-1* produced even fewer trichomes than the unwounded wild type (Fig. 1E). Unwounded wild-type plants accumulate only trace amounts of JA (Glauser et al., 2008) and our data indicate that such low doses of JA are sufficient to moderately increase trichome density. Nevertheless, JA is dispensable for trichome formation itself, as the trichome morphology of *aos* and *coi1-1* was indistinguishable from that of wild type (Fig. 1F-H). It is notable, however, that excessive MeJA treatment slightly modified trichome morphology, with increased branch number, shortened stalks and exaggerated cuticular micropapillae (Fig. 1C,D; see Table S1 in the supplementary material).

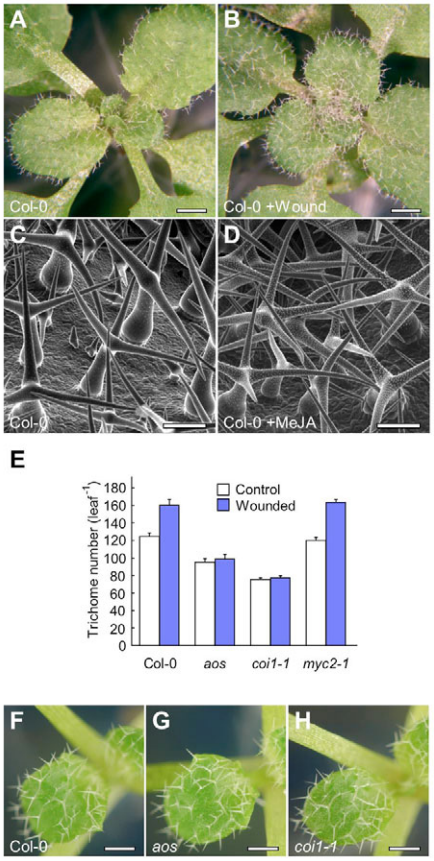

Fig. 1. Wound-induced trichome formation in *Arabidopsis* is dependent on jasmonates (JAs). (**A**,**B**) 16-DAG (days after germination) wild-type Col-0 grown under standard conditions (A) or wounded on 8 DAG (B). (**C**,**D**) Scanning electron micrograph of the adaxial epidermis of developing leaves from 10-DAG Col-0; untreated (C) or methyl jasmonate (MeJA)-treated on 6 DAG (D). Note the differences in branch numbers as well as in the texture of cell wall surface. (**E**) Quantification of trichome numbers in the fifth true leaves with or without wounding (mean±s.e. of at least 12 plants). (**F-H**) The trichome morphology of *aos* and *coi1-1* is indistinguishable from that of Col-0. Scale bars: 1 mm in A,B,F-H; 100 μm in C,D.

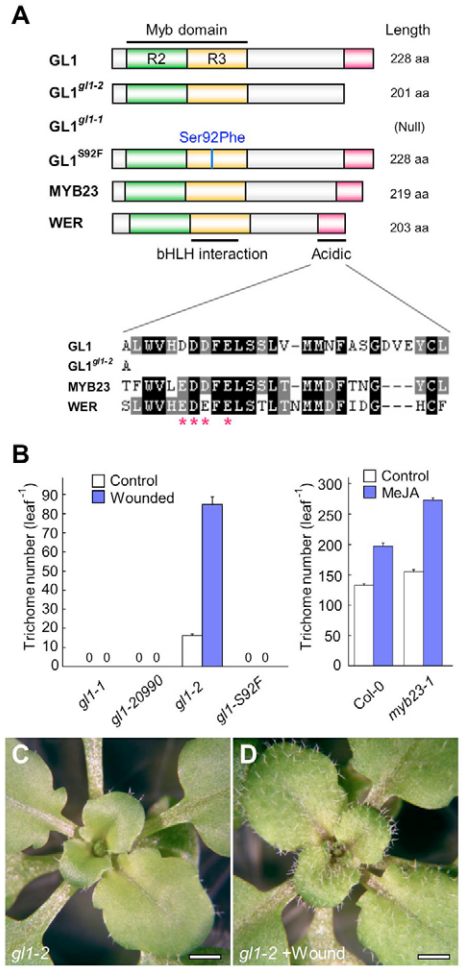

Fig. 2. Allelic series of *GL1* shows a distinct trichome-induction phenotype. (**A**) The domain structure of three paralogous R2R3-Myb proteins, GL1, MYB23 and WER, as well as of three *gl1* mutants from *Arabidopsis*. Colored boxes represent conserved domains. The bHLH-interaction motif overlaps the R3 Myb domain. The C-terminal transcriptional activator domain has a cluster of acidic residues (red asterisks). (**B**) Quantification of trichome number in *gl1* and *myb23* mutants with or without wounding (mean±s.e. of at least 12 plants). Zero indicates no trichomes produced. (**C**,**D**) 16-DAG *gl1-2* grown under standard conditions (C) or wounded on 8 DAG (D). Scale bars: 1 mm.

Trichome induction is independent of MYC2

JA signaling is divided into several branches downstream of SCF^{COI1}. To date, MYC2 is the only transcriptional activator known to act immediately downstream of SCF^{COI1} as a key regulator of the early JA-responsive transcriptome (Chini et al., 2007). However, the trichome density of the unwounded and wounded *myc2-1* mutant was comparable to that of wild type (Fig. 1E). This indicates that MYC2 is not necessary for trichome induction, and that an as yet unidentified factor(s) acts downstream of SCF^{COI1} to transduce the JA signal to trichome development.

Discrete domains of GL1 contribute to trichome induction differently

Next, we tested whether known regulators of trichome development are targets of JA signaling. GL1 and MYB23 are paralogous R2R3-Myb proteins that constitute the trichome fate-promoting Myb-bHLH-WD40 complex (Oppenheimer et al., 1991; Kirik et al., 2005). The null mutants of *GL1* (*gl1-1* and *gl1-20990*) were completely glabrous and this phenotype was not altered by wounding or MeJA treatment (Fig. 2B). Therefore, *GL1* not only regulates normal trichome development but also JA-induced trichome formation, and *MYB23* cannot act in place of *GL1*. Another paralog, WEREWOLF (WER), is expressed in the root epidermis and regulates root hair patterning (Lee and Schiefelbein, 2001).

It is reported that a weak allele of *GL1*, *gl1-2*, is normally glabrous but restores dense trichome formation in response to gamma-ray irradiation (Esch et al., 1994; Nagata et al., 1999). The reactive oxygen species (ROS) produced by water radiolysis are likely to be the inducers of trichome formation in irradiated *gl1-2*. ROS are produced in response to wounding (Orozco-Cardenas and Ryan, 1999) and we thus supposed that wound treatment would also restore trichome formation in *gl1-2*. As expected, mechanical

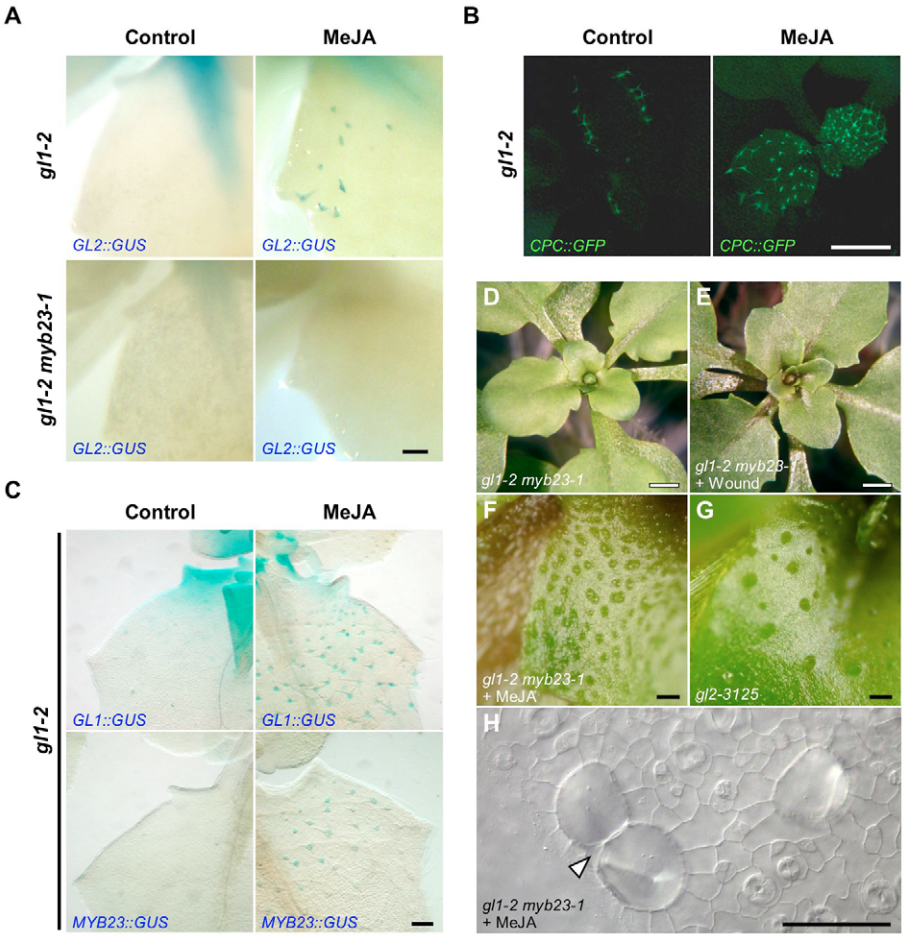

Fig. 3. *MYB23* is expressed and functions late in trichome induction. (**A**,**B**) Expression analysis of *GL2* and *CPC* in *gl1-2* mutants. MeJA treatment of *gl1-2* triggered de novo formation of trichomes marked with *GL2::GUS* (A) and *CPC::GFP* (B) expression. *gl1-2 myb23-1* lacks *GL2::GUS* expression (A). (**C**) *GL1::GUS* and *MYB23::GUS* expression in *gl1-2*. *GL1::GUS* is ubiquitously expressed in immature epidermal cells and later confined to developing trichomes, if treated with MeJA. *MYB23::GUS* is expressed in MeJA-induced trichomes but not in immature epidermal cells. (**D-H**) Epidermal phenotype of *gl1-2 myb23-1*. *gl1-2 myb23-1* is completely glabrous (D) and remains superficially glabrous after MeJA treatment or wounding (E). However, after MeJA treatment or wounding *gl1-2 myb23-1* produces numerous small bulges on the epidermis (F), which resemble arrested trichomes of *gl2* (G). Trichomes of *gl1-2 myb23-1* are frequently formed in a cluster of two to four cells (H, arrowhead). Scale bars: 100 μm in A,C,F-H; 1 mm in B,D,E.

wounding of *gl1-2* dramatically increased trichome formation (Fig. 2B-D). Double-mutant analyses indicated that this response is dependent on *AOS* and *COI1*, but not on *MYC2* or *JAR1* (see Fig. S2 in the supplementary material). Thus, wound-induced trichome formation in *gl1-2* is likely to be effected by the same JA signaling pathway as that in wild type.

GL1 directly activates the expression of multiple transcription factor genes that regulate the initiation, maturation and lateral inhibition of trichomes. Although *gl1-2* encodes a truncated protein that lacks the C-terminal transcriptional activator domain (Fig. 2A) (Esch et al., 1994; Lee and Schiefelbein, 2001), the induced trichomes of *gl1-2* had a rather normal morphology and spacing pattern (see Fig. S3 in the supplementary material), suggesting that downstream genes are active in *gl1-2*. *GLABRA2* (*GL2*) and *CAPRICE* (*CPC*) are direct targets of GL1 (Zhao et al., 2008) and we confirmed that both *GL2::GUS* and *CPC::GFP* reporters were expressed normally in the developing trichomes of MeJA-treated *gl1-2* (Fig. 3A,B). *MYB23::GUS* was also expressed in the developing trichomes of *gl1-2* (Fig. 3C), implying that MYB23 has some role in the *gl1-2* background. Interestingly, the *gl1-2 myb23-1* double mutant lost the expression of *GL2::GUS* and formed small *gl2*-like trichomes in a clustered manner, thus showing pleiotropic defects in the maturation and lateral inhibition of trichomes (Fig. 3A,D-H). It is proposed that MYB23 has a limited role in trichome development (Kirik et al., 2005); however, we find that MYB23 acts redundantly with the C-terminal domain of GL1 to activate the expression of downstream genes including *GL2*.

It is notable that *gl1-2 myb23-1* still responded to JAs and at least initiated trichomes (Fig. 3F), implicating $GL1^{gl1\text{-}2}$ (N-terminal domain of GL1) in trichome initiation. We examined the spatiotemporal pattern of $GL1^{gl1\text{-}2}$ expression using a *GL1::GUS* reporter (Larkin et al., 1993), as *gl1-2* had no mutations in the 1.4 kb upstream and 1.8 kb downstream regulatory sequences of *GL1*. In untreated *gl1-2*, *GL1::GUS* was ubiquitously expressed in immature epidermal cells (Fig. 3C; see Fig. S4A in the supplementary material), suggesting that $GL1^{gl1\text{-}2}$ is present but fails to trigger trichome initiation with a low JA level. MeJA treatment did not increase, but rather confined *GL1::GUS*-positive cells to young trichomes (Fig. 3C; see Fig. S4A in the supplementary material). Ectopic overexpression of *GL1* using the 35S promoter reduced trichome density and even blocked trichome induction by JAs (see Fig. S4B in the supplementary material) (Larkin et al., 1994). Gibberellins are proposed to activate trichome formation by upregulating *GL1* expression (Perazza et al., 1998). Our results, however, suggest that JA signaling activates the function of GL1 protein in an indirect manner. Several R2R3-Myb proteins, including GL1, share a conserved motif within their Myb domains that physically interacts with R/B-like bHLH proteins (Grotewold et al., 2000; Zimmermann et al., 2004). The *gl1-S92F* mutant, in which the Myb domain of GL1 is disrupted (Ser92Phe substitution), did not induce any trichomes in response to wounding or MeJA treatment (Fig. 2A,B). Therefore, the Myb domain of GL1 is essential for trichome initiation, and this result raised the possibility that bHLH proteins act as the activators of GL1 protein under elevated levels of JA.

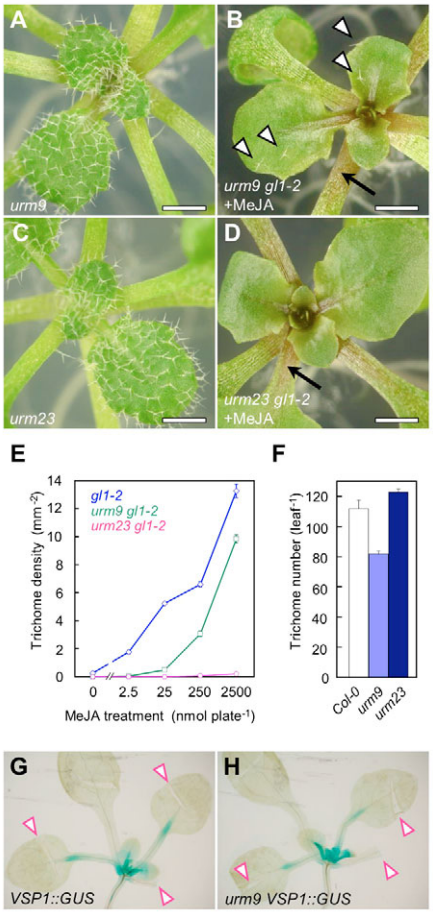

Fig. 4. Phenotypes of *Arabidopsis urm9* and *urm23* mutants. (**A**) *urm9* single mutant. (**B**) MeJA-treated *urm9 gl1-2* producing a few trichomes (arrowheads). (**C**) *urm23* single mutant. (**D**) MeJA-treated *urm23 gl1-2* with no trichomes. Note that anthocyanin synthesis is normally induced by MeJA in both *urm9* and *urm23* (B,D, arrows). (**E**) Dose-response curve of trichome density plotted against the strength of MeJA treatment. (**F**) Quantification of trichome number in *urm9* and *urm23* single mutants (mean±s.e. of at least 12 plants). (**G**,**H**) Systemic induction of JA-responsive *VSP1::GUS* expression by wounding. The wounded sites are indicated by arrowheads. Scale bars: 1 mm.

Isolation of *unarmed* (*urm*) mutants

We took a forward genetic approach to obtain clues as to the molecular link between JA signaling and GL1. We utilized the drastic trichome-induction phenotype of *gl1-2* (see Fig. S3 in the supplementary material). The M2 seedlings of ethyl methanesulfonate (EMS)-mutagenized *gl1-2* were sprayed with MeJA and glabrous plants were selected as putative mutants. In this study we analyzed two mutants, designated *unarmed9* (*urm9*) and *unarmed23* (*urm23*), in detail.

Both *urm9* and *urm23* were extragenic enhancers of *gl1-2*; *urm9 gl1-2* was strongly impaired in JA-induced trichome formation, whereas *urm23 gl1-2* completely lacked trichome induction (Fig. 4B,D,E; see Fig. S3 in the supplementary material). Backcrosses to parental *gl1-2* revealed that *urm9* and *urm23* are recessive and dominant mutations, respectively (data not shown). The JA-resistant phenotype of *urm9* and *urm23* was specific to trichomes, as they exhibited otherwise normal responses to wounding or MeJA treatment with regard to growth inhibition, accumulation of anthocyanin and systemic induction of *VSP1::GUS* expression (Fig. 4B,D,G,H). Neither *urm9* nor *urm23* showed any pleiotropic defects in the differentiation of other epidermal cell types such as root hair patterning, pigmentation of seed coat by proanthocyanidin and secretion of seed coat mucilage (see Fig. S5 in the supplementary material). Both *urm9* and *urm23* single mutants produced normal three-branched trichomes like those of the wild type (Fig. 4A,C; see Table S1 in the supplementary material). Careful examination of trichome numbers revealed that *urm9* and *urm23* had sparser and denser trichomes, respectively, than the wild type (Fig. 4F). In addition, *urm23* frequently formed trichome clusters (see Table S2 in the supplementary material). These results suggested that *URM9* and *URM23* are specific regulators of trichome patterning that are essential for JA-induced trichome formation.

urm23 is a weak allele of *TTG1*

To understand how *URM* genes link JA signaling to trichome differentiation, we used a map-based approach. *urm23* was roughly mapped to the upper arm of chromosome 5 and was completely linked with an SSLP marker, NGA139 (see Fig. S6A in the supplementary material). The *TTG1* gene is closely linked to NGA139 and was therefore regarded as a candidate for *URM23*. We sequenced the coding region of *TTG1* and identified a missense mutation converting glycine 302 to glutamate in *urm23* (see Fig. S6B in the supplementary material). We crossed *urm23* with *ttg1-213* and confirmed that they are allelic to each other (see Fig. S6C in the supplementary material). The trichome-specific phenotype of *urm23* is distinct from the pleiotropic phenotypes of known *ttg1* alleles and this might be due to the unique Gly302Glu substitution. However, we found that other *ttg1* alleles, such as *ttg1-213* and even heterozygous *ttg1-213*/+, also disrupted trichome induction (see Fig. S6D in the supplementary material). Therefore, a slight reduction of TTG1 function critically disrupts JA-induced trichome formation. TTG1 binds to GL3 and is proposed to promote physical interaction between GL1 and GL3 (Payne et al., 2000; Zhao et al., 2008). It is possible that the interaction between GL1$^{gl1\text{-}2}$ and GL3 is more sensitive to the dosage or activity of TTG1 (Larkin et al., 1999), but further experiments are needed to test this hypothesis.

URM9 encodes an Importin β family protein

Using a map-based approach, *urm9* was delimited to a 161 kb interval in the bottom arm of chromosome 2 (see Fig. S7A in the supplementary material). We sequenced all predicted gene models in this region and found two point mutations in *urm9*: one in *At2g31480* and the other in *At2g31660* (see Fig. S7A in the supplementary material). Genomic fragments of 4.1 kb and 9.3 kb containing wild-type *At2g31480* and *At2g31660*, respectively, were transformed into *urm9 gl1-2* and scored for the rescue of trichome induction. *At2g31660* alone fully restored the *urm9* phenotype (see Fig. S7B in the supplementary material). A T-DNA insertional mutant of *At2g31660* did not complement the trichome phenotype of *urm9*, indicating that these two mutants are allelic to each other (see Fig. S7C in the supplementary material). Based on these results, we concluded that *At2g31660* corresponds to *URM9*, which has been independently described as *SUPER SENSITIVE TO ABA AND DROUGHT2* (*SAD2*) (Verslues et al., 2006). The *URM9* (*SAD2*) gene encodes an Importin β family protein orthologous to mammalian Importin 7, which is also conserved in *Drosophila* (Moleskin) and yeast (Sxm1) (Merkle, 2004). The *urm9* mutation results in a severely truncated protein

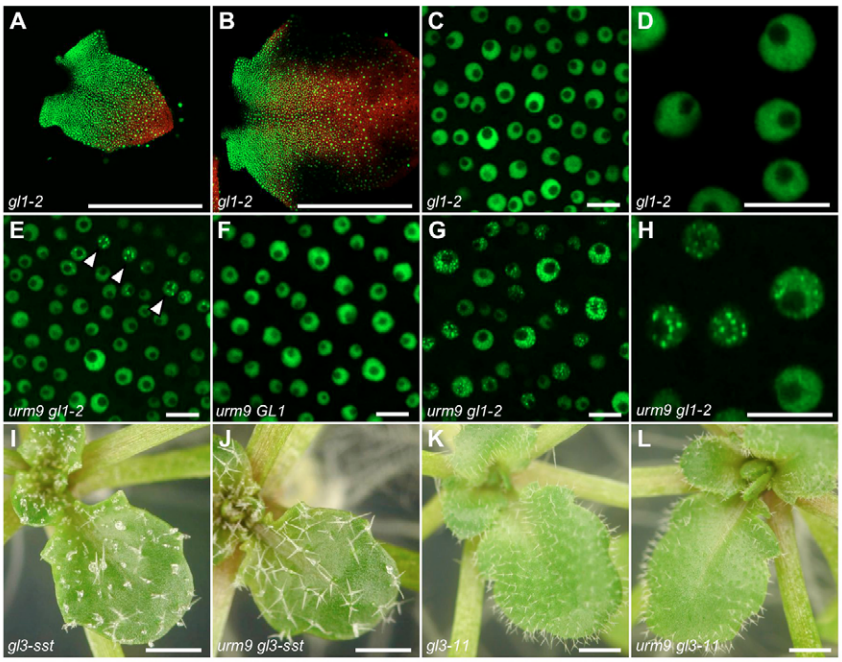

Fig. 5. *urm9* disrupts the subnuclear localization of GL3. (**A**,**B**) The overall expression pattern of *GL3::GL3-2xGFP* in the young leaf of *gl1-2* mutants. Green, GFP; red, chlorophyll autofluorescence to mark the leaf shape. The two leaves (A and B) are at different developmental stages. (**C-H**) Subcellular localization of *GL3::GL3-2xGFP* in the adaxial epidermal cells of *gl1-2* (C,D), *urm9 gl1-2* (E,G,H) or *urm9 GL1* (F). Cells shown in C,D,F-H are from leaves that are comparable in size (800 μm to 1 mm), whereas E represents a much younger leaf (400 μm). Initially, a subset of cells exhibits abnormal subnuclear distribution of GL3-2xGFP in *urm9 gl1-2* (E, arrowheads). The degree of abnormality of GL3-2xGFP distribution in *urm9 gl1-2* varies between cells, with no recognizable pattern (G,H). (**I**,**J**) Suppression of the *gl3-sst* phenotype by *urm9*. (**K**,**L**) Enhancement of the *gl3-11* phenotype by *urm9*. Trichomes are lost around the midvein in *urm9 gl3-11* (L). Scale bars: 500 μm in A,B; 10 μm in C-H; 1 mm in I-L.

owing to a premature stop codon in the sixth exon, and therefore *urm9* is most probably a null allele (see Fig. S7A in the supplementary material).

URM9 affects the subnuclear localization of GL3

Among the diverse cellular functions of Importin β family proteins, URM9 orthologs are involved in the nuclear import of various cargo proteins (Rosenblum et al., 1997; Freedman and Yamamoto, 2004; Xu et al., 2007). Notably, the loss of TTG1 (URM23) disrupts the subnuclear distribution of GL3-YFP fusion protein (Zhao et al., 2008). The phenotypic similarity of *urm23* and *urm9* prompted us to examine the subcellular localization of GL3 in the *urm9* background. We made a translational fusion of GL3 with two tandem GFPs expressed under the control of the *GL3* promoter (*GL3::GL3-2xGFP*). Consistent with the previous report, GL3-2xGFP formed subnuclear speckles in *ttg1* (see Fig. S8 in the supplementary material) (Zhao et al., 2008).

First we observed the expression pattern and the subcellular localization of *GL3::GL3-2xGFP* in MeJA-treated *gl1-2*. In young rosette leaves (smaller than 500 μm), all adaxial epidermal cells expressed GL3-2xGFP (Fig. 5A). As the leaf aged, GL3-2xGFP faded away in most cells, but scattered cells with enlarged nuclei still retained bright GFP fluorescence (Fig. 5B). This phase-change first occurred around the leaf tip and gradually shifted toward the leaf base, probably reflecting the selection of trichome precursor cells. At the subcellular level, GL3-2xGFP was evenly distributed in the nucleus but excluded from the nucleolus in *gl1-2* (Fig. 5C,D). By contrast, GL3-2xGFP exhibited a speckled subnuclear distribution in the *gl1-S92F* background, in which the GL1^{S92F} protein disrupts the interaction motif with bHLH proteins (see Fig. S6 in the supplementary material). This result is consistent with the recent report that GL3-YFP forms subnuclear speckles in the *gl1-1* null allele (Zhao et al., 2008). Conversely, the normal distribution of GL3-2xGFP in *gl1-2* suggests that C-terminally truncated GL1^{gl1-2} can interact with GL3 in vivo and regulate the subnuclear localization of GL3.

In MeJA-treated *urm9 gl1-2*, *GL3::GL3-2xGFP* was initially expressed in all adaxial epidermal cells and gradually faded away in the same manner as *gl1-2* (data not shown). In addition, GL3-2xGFP was targeted to the nucleus normally in *urm9 gl1-2*, suggesting that URM9 is not required for the nuclear import of GL3 (Fig. 5E). Instead, we found that GL3-2xGFP exhibits a speckled subnuclear localization in *urm9 gl1-2*. Although not all epidermal cells showed such a speckled distribution of GL3-2xGFP and the differences between normal and abnormal cells were continuous, the frequency of speckled nuclei tended to increase as the leaf aged. In young leaves (smaller than 500 μm) of *urm9 gl1-2*, the distribution of GL3-2xGFP was normal in most epidermal cell nuclei, and occasionally showed subnuclear speckles (Fig. 5E). In older leaves (larger than 1 mm) of *urm9 gl1-2*, a substantial proportion of epidermal cells showed abnormal GL3-2xGFP distribution (Fig. 5G,H). Nevertheless, 1 mm leaves did still actively form trichomes (see Fig. S2 in the supplementary material) and the abnormal GL3-2xGFP distribution thus correlates with a defective trichome initiation phenotype of *urm9 gl1-2*. Therefore, URM9 is likely to potentiate GL3 function by directly or indirectly regulating its subnuclear localization and this is crucial for JA-induced trichome formation. It should be noted that the subnuclear GL3-2xGFP-speckles were rarely observed in an *urm9* single-mutant background (Fig. 5F; see Fig. S10 in the supplementary material; see Discussion).

If the abnormal distribution of GL3 in *urm9* disrupts the function of GL3, one might expect *URM9* to show genetic interaction with *GL3*. A gain-of-function allele of *GL3*, *gl3-sst*, produces various distorted trichomes, resembling those produced by the strong overexpressor of *GL3* (Esch et al., 2003). As expected, the *urm9 gl3-sst* double mutant largely suppressed the *gl3-sst* trichome phenotype (Fig. 5I,J). In addition, *urm9* further enhanced the phenotype of the *gl3-11* loss-of-function allele (Fig. 5K,L), suggesting that the other GL3-homologous bHLH proteins are also under the control of URM9.

GL3 is essential for JA-induced trichome formation

Emerging evidence suggests that GL3 plays an important role in JA-induced trichome formation. The *Arabidopsis* genome contains another bHLH protein, EGL3, as a functionally redundant paralog of GL3 (Zhang et al., 2003). The *gl3-11 egl3-7* double mutant was almost completely glabrous and this phenotype was not rescued by MeJA

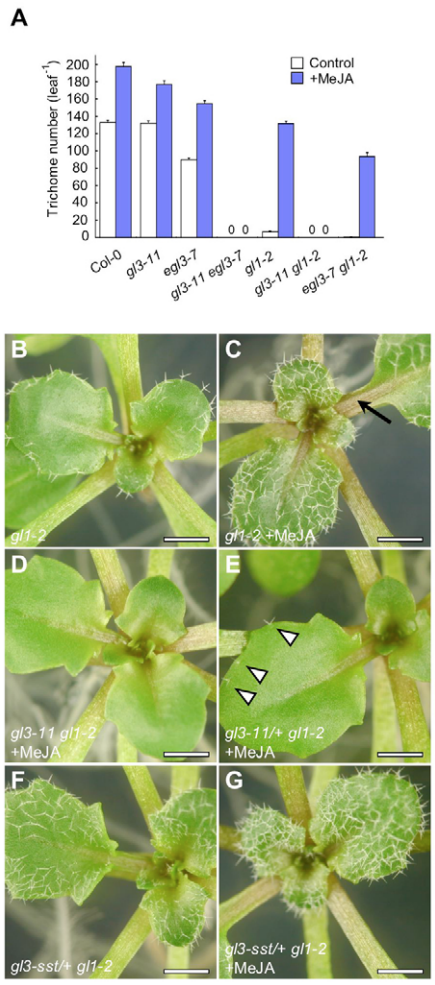

Fig. 6. GL3 is essential for JA-induced trichome formation. (**A**) Quantification of the trichome numbers in *Arabidopsis* fifth true leaves with or without MeJA treatment (mean±s.e. of at least 12 plants). Zero indicates no trichomes produced. (**B**) *gl1-2*. (**C**) MeJA-treated *gl1-2*. Note that the petioles accumulate anthocyanin (arrow). (**D**) MeJA-treated *gl3-11 gl1-2* completely lacking trichome induction. (**E**) MeJA-treated *gl3-11/+ gl1-2* with only a few induced trichomes (arrowheads). (**F**) *gl3-sst/+* partially restored trichome formation in *gl1-2* without MeJA treatment. (**G**) MeJA-treated *gl3-sst/+ gl1-2* with dense trichomes. Scale bars: 1 mm.

treatment, indicating that both GL3 and EGL3 are required for trichome induction (Fig. 6A). Next, we constructed *gl3-11 gl1-2* and *egl3-7 gl1-2* double mutants to assess the role of each bHLH in the sensitized *gl1-2* background. *gl3-11 gl1-2* was almost completely glabrous and was not responsive to MeJA treatment, whereas *egl3-7 gl1-2* produced increased trichomes in response to MeJA (Fig. 6A,D). Therefore, GL3 and EGL3 contribute to JA-induced trichome formation differently, with GL3 being the main regulator. At this point we cannot exclude the possibility that the other GL3/EGL3-related bHLH proteins also have a minor role in trichome induction because, rarely, we found that a few trichomes formed in the upper cauline leaves of MeJA-treated *gl3-11 egl3-7* (data not shown).

MeJA treatment of either *gl3-11* or *gl1-2* single mutants increased trichomes, indicating that the severe JA-insensitive phenotype of *gl3-11 gl1-2* was the result of synergistic genetic interaction (Fig. 6A). One possible explanation is that the weakly functional GL1$^{gl1\text{-}2}$ might be much more sensitive to the reduced dosage of GL3/EGL3. In support of this, MeJA treatment of *gl3-11/+ gl1-2* induced only a few trichomes, indicating that *GL3* is haploinsufficient in a *gl1-2* background (Fig. 6E). By contrast, the gain-of-function allele of GL3, *gl3-sst*, partially restored trichome formation by *gl1-2*, even in the absence of MeJA treatment (Fig. 6F,G). These results suggested that GL3 acts downstream of JA signaling and activates trichome formation in a dosage-dependent manner.

MeJA treatment enhances GL3 expression

Overexpression of *GL3* or *EGL3* results in the formation of massive trichomes (Payne et al., 2000; Zhang et al., 2003), showing that controlling the dosage of GL3/EGL3 is crucial for determining proper trichome density. We found that MeJA-treated plants had constantly stronger expression of *GL3::GL3-2xGFP* than untreated plants (Fig. 7C,D). Because GL3 is strongly expressed in developing trichomes (Zhao et al., 2008), it is possible that increased trichome formation by MeJA raises the intensity of GL3 expression. However, the induction of GL3-2xGFP fluorescence became apparent within 24 hours after MeJA treatment (Fig. 7A,B; see Fig. S9 in the supplementary material), whereas the emergence of JA-induced trichomes was first visible 48 hours after the treatment (see Fig. S3 in the supplementary material). Therefore, induction of GL3 expression is not a consequence of increased trichome number, but is an early event preceding trichome initiation.

The control of GL3 function by GL1, TTG1 and URM9 is essential for JA-induced trichome formation and we wondered whether these factors were required for the response of GL3 expression to JAs. MeJA treatment still enhanced GL3 expression in a *gl1-S92F* background (data not shown), suggesting that GL1-GL3 interaction is not required for this early response. The response of GL3 expression to JA was also intact in the *urm9* background (Fig. 7E-H; see Fig. S9 in the supplementary material), supporting our hypothesis that URM9 potentiates the function of GL3 post-translationally. In conclusion, we propose that the activation of GL3 expression by JA signaling is the key molecular event that bridges wound response to trichome development in *Arabidopsis*.

DISCUSSION

Phenotypic plasticity has long been a subject of ecological studies and many researchers have discussed the adaptive significance of induced trichome formation. Developmental analysis of trichome induction complements ecological studies in two major ways. First, it will reveal the costs and constraints of this response. Second, it will enable a cross-species comparison of the genes involved in this response. In addition, our attempts to understand the plasticity of trichome density gave us new insights into early events during trichome development, which have been overlooked in previous studies performed under non-stressed conditions.

A new branch of JA signaling in trichome development

Although the wound response of JA-related mutants has been characterized in detail, little attention has been paid to their morphological phenotypes, except for obvious male sterility and associated changes in stamen and petal development (Sanders et al., 2000; Ishiguro et al., 2001). To our knowledge, no defects in trichome formation by JA-related mutants have been reported in *Arabidopsis*. This must in part be because *coi1-1* and *aos*, two of the most severe mutants that completely lack endogenous JA function, were both generated in trichomeless strains, preventing any observation of their trichome phenotypes (Xie et al., 1998; Park et

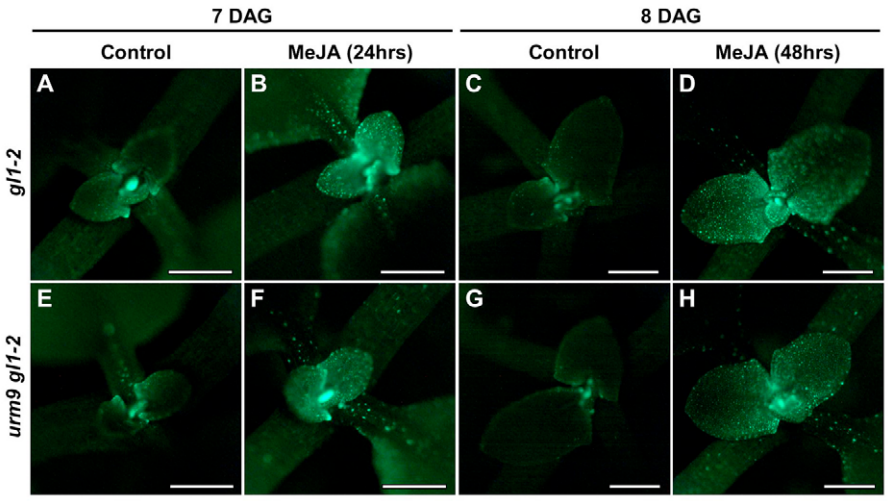

Fig. 7. MeJA-responsive expression of *GL3::GL3-2xGFP*. Epifluorescence images taken under the same setting to compare the intensity of GFP fusion expression. Shown are representative images from at least 20 plants examined. (**A-D**) *gl1-2 GL3::GL3-2xGFP*. (**E-H**) *urm9 gl1-2 GL3::GL3-2xGFP*. Plants were either left untreated (A,C,E,G) or treated with MeJA on 6 DAG (B,D,F,H). GFP fluorescence was observed and photographed on 7 DAG (24 hours post-treatment; A,B,E,F) and on 8 DAG (48 hours post-treatment; C,D,G,H). In response to MeJA treatment, both *gl1-2* and *urm9 gl1-2* induced ubiquitous GL3-2xGFP expression in the adaxial epidermis of young leaves (C,D,G,H). The difference between MeJA-treated and untreated plants was first visible 24 hours post-treatment (A,B,E,F). Scale bars: 1 mm.

al., 2002). We introgressed *coi1-1* and *aos* to wild type to restore trichomes and found that both mutants were defective in the wound-induction of trichomes. There are some reports that exogenous JA treatment increases trichome density (Traw and Bergelson, 2003; Boughton et al., 2005; Maes et al., 2008); however, our results provide the first evidence that endogenous JAs control trichome patterning in *Arabidopsis*.

The SCF^{COI1} complex targets JAZ family proteins for proteasomal degradation in the presence of JAs (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008). Overexpression of a dominant-negative form of JAZ1 recapitulated the strong JA-insensitive phenotype of *coi1*, suggesting that JAZ proteins are the primary, if not sole, targets of SCF^{COI1} (Thines et al., 2007). JAZ3 acts as a transcriptional repressor by directly binding and inhibiting the function of the bHLH-Zip transcriptional activator MYC2 (Chini et al., 2007). Since wound-induced trichome formation was dependent on COI1 but independent of MYC2, it is highly likely that an as yet uncharacterized transcriptional activator, and its cognate JAZ repressor, mediate SCF^{COI1}-dependent JA signaling and trichome differentiation. JA-isoleucine (JA-Ile) is the active ligand of SCF^{COI1} and one might wonder why the *jar1-1* mutant, which is deficient in JA-Ile conjugation, shows normal trichome induction (see Fig. S2 in the supplementary material) (Traw and Bergelson, 2003). However, the loss of JAR1 alone does not abolish all JA-amino acid conjugates (Staswick and Tiryaki, 2004), and the other weakly active ligands, such as JA-Leu or JA-Val, may be responsible for trichome induction (Katsir et al., 2008).

Notably, we found that JAs contribute to the 'default' density of trichomes in the absence of wounding. The apical meristem region constitutively expresses JA-responsive markers, even in unwounded conditions (Ellis and Turner, 2001), suggesting that young leaf primordia might contain relatively high amounts of JAs, or might be sensitized to JAs. It will be a future challenge to determine the spatiotemporal patterns of JA biosynthesis, as well as SCF^{COI1}-JAZ localization, to elucidate the unique features of this wound-independent JA response in the epidermis of developing leaf primordia.

GL3 links JA signaling to trichome development

Previous studies have established that GL3 must interact with GL1 to be recruited to chromatin and regulate the expression of downstream genes to direct trichome development (Morohashi et al., 2007; Zhao et al., 2008). This is also true for induced trichome formation because *gl1-S92F* and *urm23* (*ttg1*) mutants, both of which probably impair GL1-GL3 interaction, did not induce any trichomes after wounding or MeJA treatment. The $GL1^{gl1\text{-}2}$ protein, which lacks its C-terminal transcriptional activator domain, was still able to regulate the subnuclear localization of GL3 and to carry out the specification and initiation, but not lateral inhibition or maturation, of trichomes. This means that the downstream targets of GL1 can be divided into two classes: dependent or independent of the C-terminus of GL1. It is possible that the expression of the latter class of GL1 targets is dependent on the transcriptional activator domain of GL3 (Payne et al., 2000) and we speculate that such genes might include cell cycle regulators that direct DNA endoreduplication, the first committed step of trichome initiation. This view is supported by the finding that the loss- and gain-of-function of *GL3* specifically alter the endoreduplication cycle of trichomes (Hülskamp et al., 1994; Esch et al., 2003).

In this study, we found that MeJA treatment enhanced the expression of a *GL3::GL3-2xGFP* translational fusion in young leaves prior to trichome initiation. Since GL3/EGL3 promote trichome development in a dosage-dependent manner (Payne et al., 2000; Zhang et al., 2003), the simplest scenario is that JAs and SCF^{COI1} upregulate GL3 and thereby activate trichome initiation. During the submission of this manuscript, another group using the *GL3::GUS* promoter fusion reported that the transcriptional activity of *GL3* was induced by exogenous JAs (Maes et al., 2008). Their results are consistent with our findings using translational fusion and raise the possibility that the JA-inducible expression of GL3 is transcriptionally controlled by the 5′ promoter sequence.

The role of nucleocytoplasmic transport in trichome patterning

Our study identified the Importin 7-like protein URM9 (SAD2) as a novel regulator of GL3, adding a new layer of complexity to the control of trichome patterning. Although URM9 is ubiquitously expressed and has pleiotropic functions (Verslues et al., 2006; Zhao et al., 2007), the only known cargo of URM9 to date is MYB4, the loss of which does not induce any morphological defects (Jin et al., 2000). *urm9* did not disrupt the nuclear import of GL3-2xGFP, suggesting that URM9 does not ferry GL3 as a cargo, but affects GL3 subnuclear distribution in an indirect manner. The subnuclear GL3-2xGFP-speckles were strikingly similar to the GL3-YFP speckles reported in *gl1-1* and *ttg1-1* (Zhao et al., 2008) and it is possible that URM9 might regulate the nuclear import of GL1,

TTG1 or other GL3-binding proteins. Notably, GL3-2xGFP-speckles were prominent in the *urm9 gl1-2* double mutant, but were rarely observed in the *urm9* single mutant. Such genetic interaction can be interpreted such that the loss of URM9 attenuates the function of GL1$^{gl1\text{-}2}$ (mimicking the null *gl1-1*) and thereby disrupts the localization of GL3. However, the hypomorphic nature of *gl1-2* allele does not allow us at this point, to predict whether and how URM9 affects GL3 or GL1 (or TTG1) from genetic interaction data alone. The identification and functional characterization of the missing cargo of URM9 during trichome patterning is an important subject for future studies.

It is notable that the chromosomal location of *URM9/At2g31660* overlaps with the map position of *REDUCED TRICHOME NUMBER* (*RTN*), a major QTL controlling the natural variation in trichome density between the Col and L*er* accessions (see Fig. S7A in the supplementary material) (Larkin et al., 1996; Mauricio, 2005; Symonds et al., 2005). We are analyzing the natural variation of *URM9* sequence from multiple wild accessions of *Arabidopsis thaliana* to test whether *URM9* is the long-sought-for molecular identity of *RTN* (Y.Y. and K.O., unpublished).

Evolutionary perspectives

Wound-induced trichome formation has been reported in many plant species from diverse phylogenetic clades (Myers and Bazely, 1991; Agrawal, 1999; Traw and Dawson, 2002; Boughton et al., 2005; Holeski, 2007). It is known that the Myb-bHLH-WD40 complex is an evolutionarily conserved regulator of trichome development in Brassicaceae and Malvaceae, both of which belong to the Eurosid II clade of angiosperms (APG, 2003; Wang et al., 2004; Humphries et al., 2005; Gruber et al., 2006). Because GL3 is the key regulator of trichome induction in *Arabidopsis*, it is possible that wound-induced trichome formation in other species of Eurosid II is controlled by a similar mechanism that might have a common evolutionary origin.

By contrast, the development of glandular trichomes in Solanaceae and Scrophulariaceae, both of which belong to Euasterid I, is likely to be regulated by a distinct set of transcription factors involving MIXTA-like R2R3-Myb proteins, which do not possess a bHLH-interaction motif (Payne et al., 1999; Glover and Martin, 2000; Serna and Martin, 2006). Intriguingly, the density of glandular trichomes in tomato is increased by MeJA treatment (Boughton et al., 2005), and the tomato *jasmonic acid-insensitive1* mutant, which lacks the ortholog of *COI1*, produces fewer glandular trichomes than the wild type (Li et al., 2004), indicating that the JA signal promotes glandular trichome development in Solanaceae. The core JA signaling machinery (SCF^{COI1}, JAZ, and MYC2-related bHLH factors) is widely conserved in land plants as a key regulator of the defense response (Boter et al., 2004; Wang et al., 2005; Thines et al., 2007; Chico et al., 2008). Taken together, our study provides the novel perspective that the common wound response, as mediated by the JA signaling pathway, might have evolved multiple times to target distinct sets of epidermal cell-fate regulators, giving rise to developmentally and functionally analogous inducible defenses to cope with the threat of herbivorous insects.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/6/1039/DC1

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