**GmNAP1 is essential for trichome and leaf epidermal cell development in soybean**

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**Abstract**

Map-based cloning revealed that two novel soybean distorted trichome mutants were due to loss function of *GmNAP1* gene, which affected the trichome morphology and pavement cell ploidy by regulating actin filament assembly.

**Key message**

Trichomes increase both biotic and abiotic stress resistance in soybean. In this study, *Gmdtm1-1* and *Gmdtm1-2* mutants with shorter trichomes and bigger epidermal pavement cells were isolated from an ethyl methylsulfonate mutagenized population. Both of them had reduced plant height and smaller seeds. Map-based cloning and bulked segregant analysis identified that a G-A transition at the 3’ boundary of the sixth intron of *Glyma.20G019300* in the *Gmdtm1-1* mutant and another G-A transition mutation at the 5’ boundary of the fourteenth intron of *Glyma.20G019300* in *Gmdtm1-2*; these mutations disrupted spliceosome recognition sites creating truncated proteins. *Glyma.20G019300* encodes a *Glycine max* NCK-associated protein 1 homolog (*GmNAP1*) in soybean. Further analysis revealed that the *GmNAP1* involved in actin filament assembling and genetic information processing pathways during trichome and pavement cell development. This study shows that *GmNAP1* plays an important role in soybean growth and development and agronomic traits.

**Keywords** Soybean · Trichome · Pavement cell · *GmNAP1*

**Introduction**

Plant cells exhibit a wide variety of shapes that make important contributions to organ and tissue development and morphogenesis (Smith and Oppenheimer 2005; Yanagisawa et al. 2015). Trichomes, pavement cells, and stomata are three important components of leaf epidermal cells and play pivotal roles at each stage of development (Hegebarth and Jetter 2017). Leaf epidermal pavement cells generally have an interlocking jigsaw-puzzle shape in dicots with no protrusions or gas-exchange abilities. They protect the tissue layers located underneath, ensuring that morphologically more specialized cells are spaced out correctly; it also provides mechanical strength while still allowing growth and flexibility, and protect plants via functions such as maintaining temperature and resisting foreign invasion (Glover 2000). Stomata and trichomes are morphologically specialized (Mauricio and Rausher 1997; Serna and Martin 2006). Some flowering plants, such as tobacco (*Nicotiana tabacum*), produce multicellular trichomes, whereas others, such as Arabidopsis, have unicellular trichomes (Glover 2000). Trichomes exist on many aerial plant parts, including leaves, stems, and sepals (Huchelmann et al. 2017; Liang et al. 2014), and help to protect the plant against herbivores and insects, deter microorganisms, and maintain ion homeostasis (Schilmiller et al. 2008); for example, the trichomes in strawberry plants act as a physical barrier creating difficulties for *Chaetosiphon fragaefolii* to feed (Benatto et al. 2018). Many previous studies have proved that trichomes play an efficient role in reducing water loss through decreasing the rate of...
transpiration, on account of their barrier effect against CO$_2$ and H$_2$O exchange (Fu et al. 2013; Ning et al. 2016). Trichomes can also prevent the field spread of soybean mosaic virus (Ren et al. 2000) and increase resistance to lepidopteran insects (Hulburt et al. 2004). Flavonoid aglycones or highly methylated flavonoids biosynthesized in the trichomes also provide a chemical barrier against highly energetic and deeply penetrating UV wavelengths (Hegebarth and Jetter 2017; Oliveira and Penuelas 2002; Tattni et al. 2005).

In soybean, the surfaces of leaf, stem, petiole and pod are covered with trichomes, and they play an important role in biological and abiotic stress, such as drought tolerance (Du et al. 2009a) and pest resistance (Chang and Hartman 2017; Ortega et al. 2016). There are many soybean mutants that have been described and collected in the USDA NIL collection (Bernard et al. 1991; Bernard and Singh 1969). Bernard and Singh (Bernard and Singh 1969) reported that five loci control the different kinds of aberrant trichome phenotypes of soybean, including $P1$ (glabrous), $Pc$ (curly pubescence), $Pd$ (puberulent density), $Ps$ (puberulent sparse) and $P2$ (puberulent). $Pd1$ (puberulent density 1) and $Pd2$, have been identified to control the trichome density of soybean (Pfeiffer and Pilcher 2006). More than 50 QTLs associated with trichome related traits have been identified in soybean (Chang and Hartman 2017; Du et al. 2009b; Fang et al. 2017; Komatsu et al. 2007; Oki et al. 2011; Sonah et al. 2015; Vuong et al. 2015). $T$ locus encodes a flavonoid 3′-hydroxylase (F3′H) that controls the trichome color (Toda et al. 2002; Zabala and Vodkin 2003).

The SCAR/WAVE (suppressor of cAMP receptor/WASP family verpro lin-homologous) complex has been shown to be the major nucleator of actin filament networks in plants (Guimil and Dunand 2007; Qian et al. 2009). SCAR/WAVE proteins form a pentameric complex containing Abi (Abi-interactor), NAP (Nck-associated protein), PIR121 (p53-inducible mRNA 121), and HSPC300 (haematopoietic stem progenitor cell 300). Many mutants of the SCAR/WAVE complex have been identified in Arabidopsis, such as Grammar (gnaled)nap (El-Assal Sel et al. 2004), Pir1 (pirogi) (Li et al. 2004), sral (specifically rac 1-associated protein 1) (Basu et al. 2004), dis3 (distorted1) (Basu et al. 2005), brkl1 (brick1) (Folkers et al. 2002), and spkl (spike1) (Qiu et al. 2002). Most mutations lead to swelling and reduce branch length of trichomes and loss of interdigitation and gaps between adjacent pavement cells, and WAVE complexes are unstable in the absence of any of their members (Qian et al. 2009). Because the SCAR/WAVE complex is considered to be the only regulator of ARP2/3 (Actin-Related Protein 2/3), some mutants of the ARP2/3 complex in Arabidopsis, such as $ar2$ (Li et al. 2003), $arp3/dis1$ (Li et al. 2003), $dis2$ (El-Din El-Assal et al. 2004), and crk (cysteine-rich receptor-like kinase) (Li et al. 2003), also display very similar phenotypes in pavement cells and trichomes to those of the “distorted” mutants of the SCAR/WAVE complex.

Campbell et al. (2016) identified a fast neutron-induced the gnarled trichome mutant and mapped a 26.6 megabase interval on chromosome 20 that co-segregated with the mutant phenotype. The chromosome 20 interval included a small structural variant within the coding region of a soybean ortholog (Glyma.20G019300) of Arabidopsis Nck-Associated Protein 1 (NAP1). A wild-type soybean NAP1 transgene functionally complemented an Arabidopsis nap1 mutant. They also proved that a historic spontaneous soybean gnarled trichome mutant (T31) identified a frame shift mutation resulting in a truncation of the coding region of Glyma.20G019300. This work shows that mutation of NAP1 locus result in gnarled trichomes, however, further molecular and cellular evidence still needed to reveal its function of GmNAP1 in trichome development.

In this study, two novel Glycine max distorted trichome mutant 1–1 and 1–2 (Gmdtm1-1 and 1-2) were characterized with visibly smoother leaf, and genetic mapping proved that GmNAP1 mutations cause abnormal trichome and pavement cell development in above two mutants. The transcriptional profile analysis demonstrated GmNAP1 gene involved in actin filament assembling and genetic information processing pathways during trichome development. We further show that abnormal trichome shape and pavement size in Gmnap1 mutation involved the F-actin density in the trichome tip and the pavement cell ploidy, separately.

**Result**

**Abnormal development of trichomes and pavement cells in Gmdtm1-1 and Gmdtm1-2 mutants**

Sixteen leaf surface related mutants were obtained from EMS mutant population in our laboratory as previous described (Feng et al. 2019; Gao et al. 2017). Nine of them with hair color change, five of them with glabrous leaf, and two of them with more hair. Two of five of glabrous leaf mutants, Gmdtm1-1 and Gmdtm1-2, were studied in this paper. In contrast to the wild type plant fully covered with trichome in the young leaf and stem (Fig. 1a), these two mutants had smaller glabrous leaf and stem (Fig. 1b, c). In order to investigate the genetic relationship of Gmdtm1-1 and 1-2, they were crossed to each other, and their F1 progeny also had same phenotype as their parents. It indicated that Gmdtm1-1 and Gmdtm1-2 were allelic to each other (Fig. 1d).

The trichomes of leaves were usually straight with sharp tips in wild-type (Fig. 1h). However, the trichomes of the two mutants were not erected but drooping and had blunt tips (Fig. 1f, g, i, j). The length of the trichomes in Gmdtm1-1
and 1-2 were about 81% and 79% shorter than that of wild-type trichomes, respectively (Fig. 1h–k). The trichomes number per 2 mm² in Gmdtm1-1 and Gmdtm1-2 were 46% and 39% more than those in wild-type respectively (Fig. 1e, f, k). These results illustrated that trichome development in Gmdtm1-1 and 1-2 was dramatically different from wild-type plants in terms of size, density, and shape.

Epidermal pavement cells of wild-type Williams 82 plants were arranged in a jigsaw-puzzle pattern (Fig. 1m). The jigsaw-puzzle appearance of epidermal pavement cells was less apparent in the two alleles, Gmdtm1-1 and 1-2, than in the wild type (Fig. 1n, o). The area and perimeter length of epidermal pavement cells in Gmdtm1-1 and Gmdtm1-2 were increased comparing with Williams 82 (Fig. 1l). The pavement cell area of Gmdtm1-1 and Gmdtm1-2 was increased by 34.54% and 35.13% respectively compared with Williams 82 (see above) (Fig. 1l). In addition, the perimeter length of pavement cells of Gmdtm1-1 and Gmdtm1-2 displayed a 8.83% and 9.76% increase over that observed in Williams 82 (see above) (Fig. 1l). These results indicate that the
phenotypes of epidermal pavement cells in Gmdtm1-1 and Gmdtm1-2 were also affected.

**The Gmdtm1-1 mutation was mapped to Glyma.20G019300 gene**

To understand the inheritance pattern of Gmdtm1-1, we crossed Gmdtm1-1 with ‘Hedou 12’. The F1 plants showed a similar phenotype to the wild type, indicating that the Gmdtm1 mutation is recessive. Of 334 F2 plants analyzed, 86 showed the Gmdtm1 mutant phenotype. The ratio of the wild type and mutant type in the F2 population corresponded to the expected 3:1 segregation ratio for a single recessive gene ($\chi^2$ test, $p = 0.82$), indicating that the defect in Gmdtm1-1 behaved in a monogenic recessive manner.

To locate the Gmdtm1 locus, we used approximately 165 InDel markers between ‘Hedou 12’ and Williams 82 for mapping. The Gmdtm1-1 locus was delimited to a 0.4 Mb region between InDel markers MOL2861 (1.940 Mb) and MOL1169 (2.340 Mb) on chromosome 20 (Fig. 2a). Fifteen recombinants for the markers MOL2861 (1.940 Mb) or MOL1169 (2.340 Mb) among the F2 plants were used for further fine mapping. The Gmdtm1 locus was further pinpointed to a 0.082 Mb region between markers OL6786 (1.959 Mb) and OL6756 (2.041 Mb), containing seven annotated genes according to the Williams 82 reference genome (Glycine max Wm82.a2.v1) (Fig. 2a, Table S1). Sequencing of the 82 Kb genomic DNA region containing these seven genes revealed that only Glyma.20G019300 gene had a G-to-A change in 3959 bp between Williams 82 and Gmdtm1-1 mutant, while no sequence difference was detected in the other six genes. Transcripts analysis of Glyma.20G019300 gene indicated that there was a 10 bp deletion in the seventh exon of Gmdtm1-1 mutant comparing Williams 82 (Fig. 2b, c). Further analysis indicated that the G-to-A change of Glyma.20G019300 disrupted the splice acceptor site and created a new splicing acceptor site at 10 bp downstream of the mutation site. In addition, the 10 bp deletion in the CDS resulted in a frameshift and a premature stop codon to produce a putative truncated protein lacking 1133 amino acid residues of the carboxyl

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**Fig. 2** Positional cloning and characterization of the Gmdtm1 locus. 

(A) Mapping of the Gmdtm1 locus. The Gmdtm1 locus was delimited to a 0.4 Mb region (in green color) between InDel markers MOL2861 and MOL1169 on chromosome 20 and further to an 82 kb region (in green color) bounded by markers OL6786 and OL6756. 

(B) Schematic structure of Glyma.20G019300 gene and mutant alleles. 

(C) A 10 bp deletion of Gmdtm1-1 transcript comparing with Glyma.20G019300 transcript. 

(D) The predicted CDS length of Glyma.20G019300 and Gmdtm1-1 was 4167 and 957 bp, respectively.
region (Fig. 2c, d). This mutation of Glyma.20G019300 may be responsible for the phenotype of Gmdtm1-1.

**The Gmdtm1-2 mutation was also mapped to Glyma.20G019300 gene**

Another distorted trichome mutant, Gmdtm1-2, was discovered from the same EMS mutagenesis population as above. The morphology of Gmdtm1-2 was very similar to that of Gmdtm1-1. Genetic analysis indicated that there were 89 mutant plants segregating from 289 progeny plants of the heterozygous Gmdtm1-2 plants. The ratio of the mutant and wild type in this population was in accordance with the expected 1:3 distribution. This indicates that Gmdtm1-2 is a single recessive mutant.

To investigate which gene contributed to the distorted trichome phenotype in Gmdtm1-2, we re-sequenced the genome of the mutant and wild-type pools from the Gmdtm1-2 M2 segregating population and calculated a SNP index using the BSA method (see details in “Materials and methods” section). A total of 8866 SNPs were detected from index using the BSA method (see details in “Materials and methods” section). A total of 8866 SNPs were detected from the two pools after filtering and used to plot the chart. A link-methods section). A total of 8866 SNPs were detected from index using the BSA method (see details in “Materials and methods” section). A total of 8866 SNPs were detected from the two pools after filtering and used to plot the chart. A link-methods section). A total of 8866 SNPs were detected from index using the BSA method (see details in “Materials and methods” section).

To elucidate the pathway leading to the abnormal development of epidermal trichomes and pavement cells in Gmdtm1-1, we performed RNA-seq of Gmdtm1-1 and Williams 82 using Illumina sequencing technology at the V2 growth stage when the second trifoliate leaf was fully opened. The number of clean reads obtained from the raw reads ranged from 13,581,461,245 to 16,768,218,200 bp in the six samples (Table S2). About 85.01% of reference genes were detected. A total of 3040 genes (including 3834 transcripts) were up-regulated and 2061 genes (including 2858 transcripts) were down-regulated in Gmdtm1-1 compared with Williams 82. The qRT-PCR results were consistent with the data derived from RNA-seq, demonstrating the reliability of our RNA-seq results (Fig. S1a, b). All DEGs (Differential Expression Genes) were mapped to the KEGG (https://www.kegg.jp) and 121 KEGG pathways were involved. Moreover, 31 pathways were identified with significant enrichment of DEGs (Fig. 5a).

The actin cytoskeleton of plant plays an important role in cell development, cell morphogenesis, and the establishment and maintenance of cell polarity. RACs,
WAVE complex and Arp2/3 complex participate in the synthesis of the actin filament together (Yalovsky et al. 2008). We identified 20, 27 and 19 genes relating RACs, WAVE complex and ARP2/3 complex respectively (Table S5). According to the result of transcriptome analysis, 3 DEGs (Glyma.05g035200, Glyma.07g203100, Glyma.12g208000) which encode RAC protein were significantly up regulated compared with wild type.
Two DEGs (Glyma.07G221000, Glyma.20G019300) and its homologous gene, GmNAP1, are indicated by black arrows. Conserved gene pairs between the segments are connected by lines. The red arrow represents the anchor GmNAP1 gene, and the gray arrow represents the anchor Glyma.07G221000 gene, and the gray arrow represents the anchor Glyma.07G221000 gene. The flanking genes around GmNAP1 and Glyma.07G221000 are indicated by black arrows. Conserved gene pairs between the segments are connected by lines. e Expression profiles of the Glyma.20G019300 (GmNAP1) and its homologous gene, Glyma.07G221000.

**GmNAP1 might affect the genetic information processing to regulate cell size of pavement cell**

The transcriptome analysis identified 150 DEGs, including 136 down-regulated genes and four up-regulated genes, involved in pathways associated with “replication and repair,” “translation,” and “transcription,” which included 12 sub-pathways (Fig. S3b). Among the 150 DEGs between Gmdtm1-1 and Williams 82, 108 DEGs (94 down-regulated and 14 up-regulated) were related with ‘replication and repair’ pathways (Table S3a). Of these, 27 DEGs (3 up-regulated and 24 down-regulated) were enriched in the “DNA replication” pathway (Fig. S3b, Table S3a). Genes associated with the DNA replication pathway, such as DNA polymerase α-primase complex, δ complex, ε complex, MCM complex, clamp loader complex, and helicase, were all down regulated (Table S3a). We also found 27 DEGs enriched in the “mismatch repair” pathway. The GO enrichment analysis also indicated that “DNA replication” and “transcription” were significantly enriched (Fig. S4). These genes, such as MutL, MutS, RFC, Exonuclease, DNA polymerase δ, and DNA ligase I, were down regulated (Fig. S3a), which was probably related to the pleotropic phenotypes to Gmdtm1-1 and Gmdtm1-2.

The DNA contents of the mature pavement cells of wild-type and mutant were measured by flow cytometry to evaluate the effects of GmNAP1 during genetic information processing. The ratio of diploid cells of Gmdtm1-1 (52.75 ± 1.17 percentage of total cells) was significantly less than that of Williams 82 (66.18 ± 5.31 percentage of total cells). By contrast, the percentage of tetraploid cells of Gmdtm1-1 (29.08 ± 1.04 percentage of total cells) increased significantly compared with Williams 82 (20.38 ± 1.78 percentage of total cells) (Figs. 5f, S3c). Increased DNA content or polyploidization is usually associated with increased cell size (Frawley and Orr-Weaver 2015; Orr-Weaver 2015).

Therefore, the result suggests that the increased ratio of polyploidy cell of Gmdtm1-1 might lead to the enlarge pavement cell.

**Gmdtm1 also affected plant height and yield**

Gmdtm1 also showed the defects in plant growth and yield beside abnormal trichome development. Gmdtm1-1 and I-2 had reduced height and smaller seeds. The mean seed area in Gmdtm1-1 and I-2 was 28.21 ± 1.09 and 26.28 ± 2.33 mm², respectively, which was decreased by 31.51% and 36.20% compared with Williams 82 (41.19 ± 2.31 mm²) (p < 0.05) (Fig. 6a, c). The seed circumference in the two mutants was 18.99 ± 0.39 and 18.45 ± 0.85 mm, which was decreased by 20% and 21% compared with that of Williams 82 (19.91 ± 1.20 mm) (p < 0.05) (Fig. 6b). The plant yield in the two mutants was 7.94 ± 1.88 and 8.56 ± 1.43 g, respectively, which was significantly lower than Williams 82 (126.08 ± 4.36 cm) (p < 0.05) (Fig. 6e). The various right and development phenotypes of Gmdtm1 reveals...
that GmNAP1 is required for soybean growth and agronomic traits.

Discussion

The SCAR/WAVE complex is involved in many processes contributing to important crop traits, such as stomatal dynamics and water use efficiency, infection thread formation during root nodulation, and control of cellular growth that impacts organ architecture and the adhesive properties of cells in the context of a tissue (Deeks et al. 2004; El-Assal Sel et al. 2004; Fu et al. 2013; Li et al. 2004; Ning et al. 2016; Zhou et al. 2016). The component of SCAR/WAVE complex, NAP (Nck-associated protein), has been reported to regulate actin-based cell morphogenesis and multiple developmental processes in Arabidopsis (Brembu et al. 2004; Deeks et al. 2004; El-Assal Sel et al. 2004; Fu et al. 2013; Li et al. 2004; Ning et al. 2016; Zhou et al. 2016). In rice, less pronounced lobe epidermal cell3-1 (lpl3-1),
encoding NCK-associated protein 1, developed a smooth surface, with fewer serrated pavement cell (PC) lobes, and decreased papillae (Zhou et al. 2016). DS8 (Drought Sensitive 8) gene, a NAP1-like protein in rice, recently was reported to affect drought sensitivity by involvement leaf epidermal development and stomatal closure (Huang et al. 2019). In soybean, Campbell et al. (2016) identified a 26.6 megabase interval on chromosome 20 that co-segregated with the gnarled trichrome phenotype in a fast neutron mutant population. This chromosome 20 interval included a small structural variant within the coding region of a soybean NAP1 locus. A wild-type soybean NAP1 transgene functionally complemented an Arabidopsis nap1 mutant. In this study, two EMS induced soybean trichrome mutants (Gmdtm1-1 and 1-2), were isolated and mapped to Glyma.20G019300 gene independently by map-based cloning. The soybean transgenic complementation experiment clearly proved the function of GmNAP1. This work not only confirmed the previous results, but also dispelled doubts of its function because of tortuous genetic background of previous mutant. The WAVE/SCAR complex and ARP2/3 complex are important protein complexes belonging to the ROP small GTPase signal transduction pathway (Vernoud et al. 2003; Yanagisawa et al. 2013; Zhang et al. 2008), which promote actin polymerization by enhancing F-actin nucleation and side-binding activities that result in the initiation of fine actin filaments (Hulskamp 2004). In Gmdtm1-1 mutant, 5 DEGs relating to WAVE complex were also found in this study (Tab. S5). The further characterization functions of these genes will help to resolve the contribution of SCAR/ WAVE complex to soybean agronomic traits.

Epidermal pavement cells of most dicot flowering plant species have lobed morphologies (Smith and Oppenheimer 2005), and the actin filament plays a critical role in the spatial regulation of pavement cell growth (Pratap Sahi et al. 2017). Lobe initiation and outgrowth of the pavement cell appear to require cortical fine actin microfilaments localized to sites lacking well-ordered cortical microfilaments (Armour et al. 2015; Frank and Smith 2002; Fu et al. 2005). Actin filaments can be assembled both outside and inside the nucleus and may be involved in chromatin remodeling and transcriptional control (Olave et al. 2002). Actin filaments assembled outside the nucleus support the overall shape of the cell and aid in cellular organization, while actin filaments assembled inside the nucleus respond to multiple cellular perturbations, including heat shock, protein misfolding, integrin engagement, and serum stimulation (Belin et al. 2015). In our study, we found that the

Fig. 6 Yield indices of Williams 82, Gmdtm1-1, and Gmdtm1-2. a Seed phenotype of Williams 82, Gmdtm1-1, and Gmdtm1-2. Bars = 1 cm. b Weight of 100 seeds and seeds number per plant of Williams 82, Gmdtm1-1, and Gmdtm1-2. c Seed area and seed perimeter of Williams 82, Gmdtm1-1, and Gmdtm1-2. d Yield per plant of Williams 82, Gmdtm1-1, and Gmdtm1-2. Data are presented as mean ± SD. e Plant height of Williams 82, Gmdtm1-1, and Gmdtm1-2. Data are presented as mean ± SD.
lobe and neck structures of pavement cells were nearly absent in the two mutants, *Gmdtm1-1* and *Gmdtm1-2*, especially the lobe. The reduced F-actin in the tip trichome (Fig. 5b) and increased ratio of polyploidy cell of *Gmdtm1-1* might closely related to the abnormal cells size and shape of both pavement cell and trichrome. Besides the pavement cell and trichrome, the plant height and seed size were also altered in mutant. We also noticed pathways, associated with “lipid metabolic process”, “fatty acid metabolic process” and “fatty acid biosynthetic process”, were significantly enriched in the GO enrichment analysis (Fig. S3a, Table S4). Further analysis of more factors different pathways related to *GmNAP1* will help to improve soybean varieties in the future.

**Materials and methods**

**Plant materials**

Seeds of soybean (*Glycine max*) cultivars Williams 82 and ‘Hedou 12’ were obtained from Chinese Academy of Agricultural Sciences and Jining Academy of Agricultural Sciences, respectively. *Glycine max distorted trichome mutant 1–1* and *1–2* (*Gmdtm1-1* and *Gmdtm1-2*) were identified from an ethyl methylsulfonate (EMS) mutagenized population of Williams 82 (Feng et al. 2019). The plant heights were measured at R8 stage; the seed number per plant, weight of hundred seeds, seed area and seed perimeter was measured after harvesting.

**Genetic mapping and bulked segregant analysis (BSA)**

F2 plants derived from a cross between the mutant and ‘Hedou 12’ were used for mapping. Plants with distorted trichome phenotype were selected for preliminary mapping with about 165 InDel (insertion or deletion) markers between ‘Hedou 12’ and Williams 82 (Song et al. 2015). Fine-mapping oligos were developed using data from the whole-genome re-sequencing of ‘Hedou 12’ (Song et al. 2015), and differentially expressed genes (DEGs) were selected by using the criteria $q < 0.05$ and $\log_2$ (fold change) $\geq 1$. Reverse-transcription PCR was performed using a PrimeScript RT-PCR Kit (Takara, RR014) following the manufacturer’s methods. The samples used for qPCR were the same as the RNA-seq, which has three independent biological replicates, and the genes relative expression level were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *Cons4* (*Glyma.12G020500*) (Libault et al. 2008).

**Phylogenetic analysis**

The amino acid sequence of *GmNAP1* was used to identify homologous genes of *GmNAP1* in Phytozome (https://phytozone.jgi.doe.gov). A neighbor-joining tree was generated with the Poisson correction method using MEGA 7.0 software (Kumar et al. 2016). Bootstrap replication (1000 replications) was used to determine statistical support for the nodes in the phylogenetic tree. Microsynteny analysis was performed using MCSanX (Wang et al. 2012). Gene structure was analyzed as previous work (Dai et al. 2018).

**RNA-seq analysis and qPCR validation**

Total RNA was isolated from young leaves using TRIzol, following the manufacturer’s methods (Invitrogen, Carlsbad, USA). Paired-end sequencing libraries with an insert size of approximately 350 bp were sequenced on an Illumina Hiseq X Ten platform at Novogene Biotech Company (Beijing, China). Sequences were deposited at the National Center for Biotechnology Information (NCBI) with the accession number SRP149402. Gene expression (FPKM, fragments per kilobase of transcript per million fragments mapped) levels were estimated using the Cufflinks software (version v 2.1.1) (Trapnell et al. 2012), and differentially expressed genes (DEGs) were selected by using the criteria $q < 0.05$ and $\log_2$ (fold change) $\geq 1$. Reverse-transcription PCR was performed using a PrimeScript RT-PCR Kit (Takara, RR014) following the manufacturer’s methods. The samples used for qPCR were the same as the RNA-seq, which has three independent biological replicates, and the genes relative expression level were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *Cons4* (*Glyma.12G020500*) (Libault et al. 2008).

**Scanning electron microscopy (SEM)**

Mature leaves were cut into 1 cm squares, and fixed in 2.5% glutaraldehyde solution for SEM analysis (Zhou et al. 2016). The SEM images were acquired using HITACHI S-3400 and JEOL JSM-IT500. Pavement cell area and perimeter length, and trichome length were measured using ImageJ software (Schneider et al. 2012). Measurements of trichome density with three biological repeats were performed using images with 2 mm$^2$ (2 × 1 mm). Trichome lengths with three biological repeats were measured for 10 trichome cells from each plant. pavement cell...
areas and perimeter lengths were measured with three biological repeats using 20 epidermal cells from each plant.

**Actin cytoskeleton and Flow cytometry analysis**

The actin staining performed as previously described (Zhou et al. 2016). The trichome of Williams 82 and GmdtmI-1 at VC stage were stained with iFluor 488 phalloidin, and the fluorescence images were projections of confocal sections (C2, Nikon). The integrated fluorescence intensity of transverse sections with 6 repeats taken at the top of the trichome using NIS Elements software (version 4.6). The core fluorescence was a wide ring around the perimeter from the trichome surface which occupied half area of transverse section.

The flow cytometry analysis was followed by the previous studies (Dolezel 1991; Dolezel and Bartos 2005). All samples were analyzed by flow cytometry (LSRFortessa, BD), FACSDiva software (version 6.1.3) and FlowJo software (version 10.6.1).

**Statistics analysis**

All experiments were carried out using at least three biological repeats for each treatment and all statistical analyses were performed with R software (version 3.6.2). Pairwise-comparison was performed by Student’s t-test. Asterisks indicate significant differences as determined (*p < 0.05; **p < 0.01; ***p < 0.001). Multiple comparison tests were performed with multcomp R-package. Significance level was set at p < 0.05.

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**Author contributions**  SY and XF designed the research; KT, XXF, TW, YZ, JM and JL performed experiments; YZ, and HY helped with data analysis; HZ and KT performed bioinformatics analyses; KT, SY and XF wrote the manuscript.

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