HlMIXTA1 Is a Candidate Gene for Glandular Trichome Initiation in Hop (*Humulus lupulus* L.)

Ryuji Sugiyama, Haruka Oda*, Yasumasa Takao** and Yoshiya Murakami**

Abstract
Hop（*Humulus lupulus* L.）is an important material for beer brewing. Hop bitter resins and essential oils are biosynthesized and accumulated in lupulin glands in the female flowers of hop. Lupulin glands are large glandular trichomes that consist of four basal cells, four stalk cells and a large, flattened, one-layered, glandular head. The glandular head consists of 100–200 cells. The development of non-glandular trichomes has been well studied using the model plant *Arabidopsis thaliana*. However, glandular trichome development is not well analyzed. One of the essential genes for trichome initiation is the R2R3 *MYB* gene. We isolated three *MYB* genes, *HlMYB1*, *HlMYB2* and *HlMIXTA1*, from female flowers of hop. Spatial and temporal expression of these three *MYB* genes was studied using *in situ* hybridization. *HlMIXTA1* was expressed in the early stage of trichome development in female flowers. *HlMIXTA1* is a candidate for glandular and non-glandular trichome initiation in hop.

Key words: *MYB* gene, hop, glandular trichome, *in situ* hybridization

Introduction

Hop（*Humulus lupulus* L.）is a crucial ingredient for beer brewing. Hop female flowers accumulate secondary metabolites that give beer its bitter taste and aroma. These secondary metabolites also exhibit interesting bioactive properties; for example, prenylflavonoids and hop bitter acids have cancer preventive potentials[1]. These secondary metabolites are mainly accumulated in glandular trichomes（hairs）on bracteoles of the hop female flower. The glandular trichomes are called lupulin glands. Glandular trichomes also exist in the other plant parts of hop, except the root. Hop has three types of glandular trichomes, two peltate and one bulbous type（Fig. 1）[2]. The lupulin gland is one of the peltate trichomes that are found on the female flowers. Glandular trichomes initiate by hypertrophy and anticlinal division of a protodermal cell, followed by establishment of the glandular head cells. Lupulin glands consist of four basal cells, four stalk cells and a large, flattened, one-layered, glandular head[3]. The glandular head of lupulin glands consists of 100–200 cells. Lupulin glands become biconical in shape after the accumulation of secreted materials.

Trichome development has been well studied using the model plant *Arabidopsis thaliana*. Several genes that are necessary for trichome development have been identified. A mutation of a R2R3-MYB gene *GLABRA1*（*GL1*）results in glabrous（hairless）plants in *A. thaliana*[4]. In addition to the R2R3-MYB gene, the basic helix–loop–helix（bHLH）factors and the WD40 repeat（WDR）protein play a crucial role in trichome development[5]. However, *A. thaliana* has only non-glandular trichomes and not glandular trichomes, so the development of glandular trichomes has been studied using tobacco[6] and tomato[7]. Liu et al. showed that the C2H2 zinc-finger transcription factor gene plays an important role in glandular trichome development in tobacco[6]. The jasmonic acid signaling pathway regulates tomato glandular trichome development[7]. However, the functions of the MYB gene family in glandular trichome development remain unclear.

In this study, to isolate *MYB* genes that might be involved in the development of glandular trichomes in the hop plant, we used degenerate primers and isolated three *MYB* genes:

*Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama
**Experimental Station for Medicinal Plant Research, School of Pharmacy and Pharmaceutical Science, University of Toyama

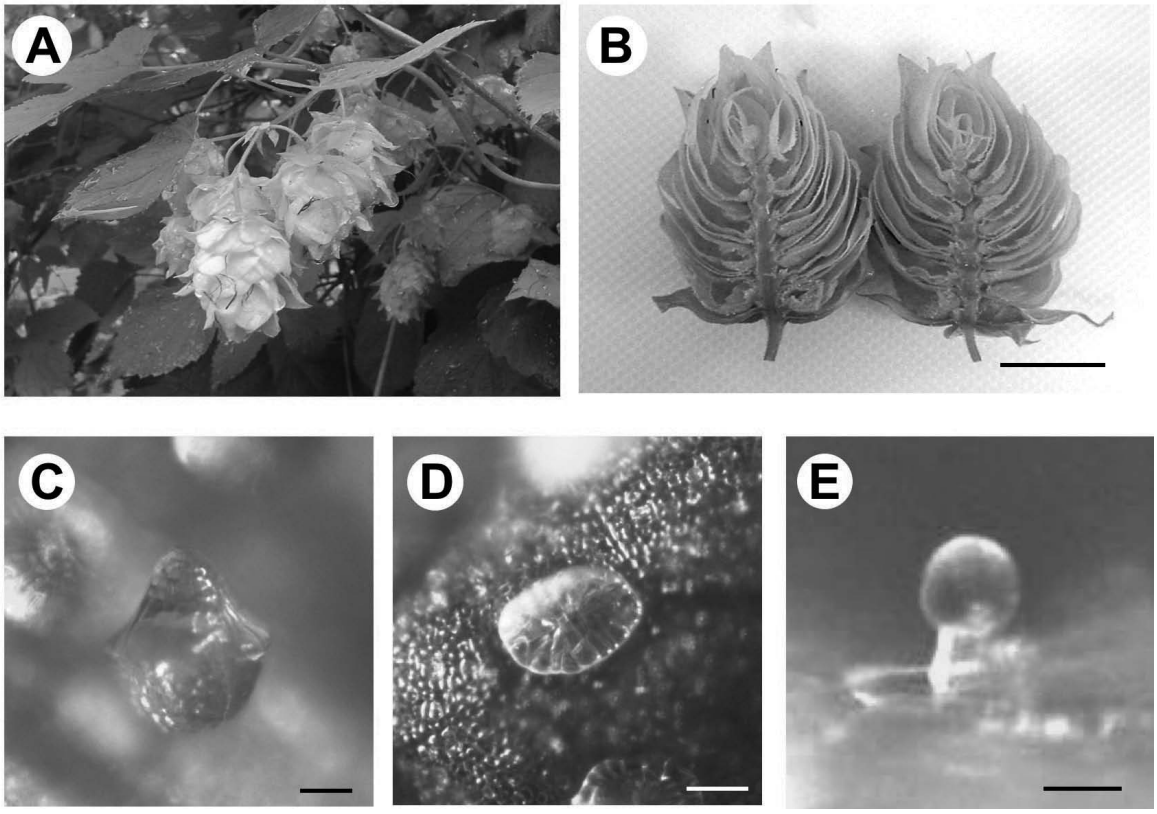

Figure 1. Hop female flower and glandular trichomes.

A. Hop female flowers. **B.** Transverse section of female flower. **C.** Mature lupulin gland on the abaxial side of a bracteole of female flower. **D.** Peltate trichome on the abaxial leaf surface. **E.** Bulbous trichome on the adaxial side of a bracteole of female flower. Scale bars: 1 cm (B), 50 μm (C, D), 25 μm (E).

HlMYB1, *HlMYB2*, *HlMIXTA1*. The spatial expression patterns of these genes in the early stage of female flowers were studied using *in situ* hybridization (ISH).

Materials and Methods

Plant material

Female plants of hop (*H. lupulus* L.) were grown in the herb garden of Toyama University in Japan. Young female flowers were collected in June 2004, 2005, and 2006. The average temperature in Toyama was 22.0℃ in 2004, 22.8℃ in 2005, and 21.4℃ in 2006.

Isolation of MYB genes

The total RNA from 3-mm hop flowers was isolated using a RNeasy Plant Mini Kit (Qiagen). mRNA was isolated using the PolyA Tract mRNA isolation system III (Promega, Madison, WI) and reverse-transcribed using a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). Polymerase chain reaction (PCR) was performed according to a method described previously[8] using the R2R3 MYB domain degenerated primers (5'-GTG GGA ARA GTT GYM GRY TRA GRT GG-3', 5'-GTT CCA RTA RTT CTT BAY YTS RTT-3') that were designed from the *A. thaliana GL1* gene, *Cucumis sativus* werewolf gene, *Perilla frutescens MYBC05* gene, and *Gossypium hirsutum GhMYB109* gene. PCR products were separated on 1% (w/v) agarose gels. DNA fragments of 180 bp were purified with Amicon Microcon PCR centrifugal filter devices (Millipore, Billeria, MA) and were cloned into a pCR 2.1-TOPO cloning vector using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Positive clones were identified with colony PCR and hybridization using a partial sequence of the MYB domain from the *A. thaliana GL1* gene as a probe. The clones were sequenced using a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 DNA sequencer (Applied Biosystems). The partial sequences were used to isolate the full-length genes using the GeneRacer Kit (Invitrogen) and the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA).

Reverse-transcription polymerase chain reaction (RT-PCR)

Poly (A)$^+$ RNA was isolated using the PolyATract mRNA isolation system (Promega), and 100 ng poly(A)$^+$ RNA was used to generate cDNA with the First-Strand cDNA synthesis kit (Amersham Biosciences) using NotI-d(T)18 as a primer. The synthesized first-strand cDNA was used as a template for PCR. Actin was used as a control. The primers used were the same sets employed in the cloning of 3' and 5' fragments of each MYB-like gene. The primers used for RT-PCR are shown in Table 1. The PCR conditions for *HlMYB1* and *HlMYB2* were 94℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 68℃ for 30 s, and 72℃ for 30 s. The PCR conditions for *HlMIXTA1* were 94℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 64℃ for 30 s, and 72℃ for 2 min. The PCR conditions for *HlActin* were 94℃ for 5 min followed by 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s.

DNA blot analysis

Genomic DNA was extracted from leaves by using a Nucleon PhytoPure kit (Amersham Biosciences, Buckinghamshire, UK). For DNA blot analysis, genomic DNA was digested overnight at 37℃ with restriction enzymes, then 15 μg digested DNA was electrophoresed on 1% agarose gels and blotted onto Immobilon-Ny+ membranes (Millipore, MA, USA). The membranes were hybridized with PCR products corresponding to the gene-specific region using a Thermoblock rotator (SN-06BN; Nissin Scientific Corp., Tokyo, Japan). Labeling and detection were performed using AlkPhos direct nucleic acid labeling and detection systems (Amersham Biosciences), according to the manufacturer's instructions.

In situ hybridization

Flower buds of female hop plants were fixed for 1 hr at room temperature in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde. The fixed tissues were dehydrated with a graded ethanol series and embedded in Histosec (Merck, Darmstadt, Germany) then sliced into 7-μm sections and attached to MAS-coated glass slides (Matsunami Glass, Osaka, Japan). Paraffin was removed by a xylene and ethanol series. The primers used for isolation of a gene-specific region are shown in Table 1 (HlMIXTA1-ISH-F and HlMIXTA1-ISH-R). Digoxigenin (DIG)-labeled antisense RNA probes were prepared from the PCR products of a gene-specific clone using T7 or SP6 RNA polymerase (Roche Diagnostics, Mannheim, Germany). Tissue sections were hybridized with a probe at 65℃ for 16 h, then washed in a solution containing 0.2× saline sodium citrate buffer (SSC) for 2 h at 65℃ followed by 0.2× SSC at room temperature for 5 min. The hybridizing probe was detected using an anti-DIG conjugated alkaline phosphatase (Roche Diagnostics). Photographs were taken with a bright-field microscope (Olympus, Tokyo, Japan) and CCD camera (Polaroid, MA, USA).

Results

Three *MYB* genes from female hop flower buds

To isolate the MYB gene family, RT-PCR was performed using degenerated primers designed from conserved regions of *MYB* genes (*A. thaliana GL1* gene, *C. sativus*

Table 1 Gene-specific primers

Name	Sequence
HlMYB1-F	5' -AAC TAC CTC AGA CCT GAC CTC AAA CG-3'
HlMYB1-R	5' -GGT GGT GGT GGT TGT TGT GGC GG-3'
HlMYB2-F	5' -CTA CAT TCA GCT TCA TGG TGA AGG-3'
HlMYB2-R	5' -ACC ACC ACC ATC TCG ACC AAG CTC TAG G-3'
HlMIXTA1-F	5' -GCA ACT CAC TTG CCA AAG AGA ACA GAC AAC-3'
HlMIXTA1-R	5' -ACC ACT GAG ACC AGC ACA CTA CCA C-3'
HlActin-F	5' -GAC CTT GCT GGG CGT GAT-3'
HlActin-R	5' -TTG GAA GAG GAC TTC AGG GC-3'
HlMIXTA1-ISH-F	5' -AAT CAA ACT ACT ACT ACG ACA GTT GGT GGT GGA-3'
HlMIXTA1-ISH-R	5' -GCA GCT TCC GTC GTT AGA GTT CTC TCC-3'

werewolf gene, *P. frutescens MYBC05* gene, and *G. hirsutum GhMYB109*). Three R2R3 MYB-like fragments were isolated. These isolated sequences showed high similarity with *AtMYBWER*, *AtMYB4* and *AmMIXTA*. Using these fragments, full-length cDNA was isolated by 3' and 5' RACE. The full-length sequence of the *AtMYBWER*-like fragment was not isolated in this research. Two MYB-like genes were isolated and named as *HlMYB1* and *HlMYB2* by 3' and 5' RACE of the *AtMYB4*-like fragment. The full length of the *HlMYB1* gene was 795 bp, and the putative amino acid sequence was 265 aa (accession number: AB292244). The full length of the *HlMYB2* gene was 783 bp, and the putative amino acid sequence was 261 aa (accession number: AB292245). We isolated a 1392-bp full-length sequence by 3' and 5' RACE of the *AmMIXTA*-like fragment. The putative amino acid sequence of this sequence was 464 aa. A BLAST search of the GenBank protein database revealed that this gene has 91% similarity with *AmMIXTA* and designated *HlMIXTA1* (Fig. 2; accession number: AB292243). Southern analysis using the gene-specific region as a probe showed that the hop genome has two copies of *HlMIXTA1* (Fig. 3).

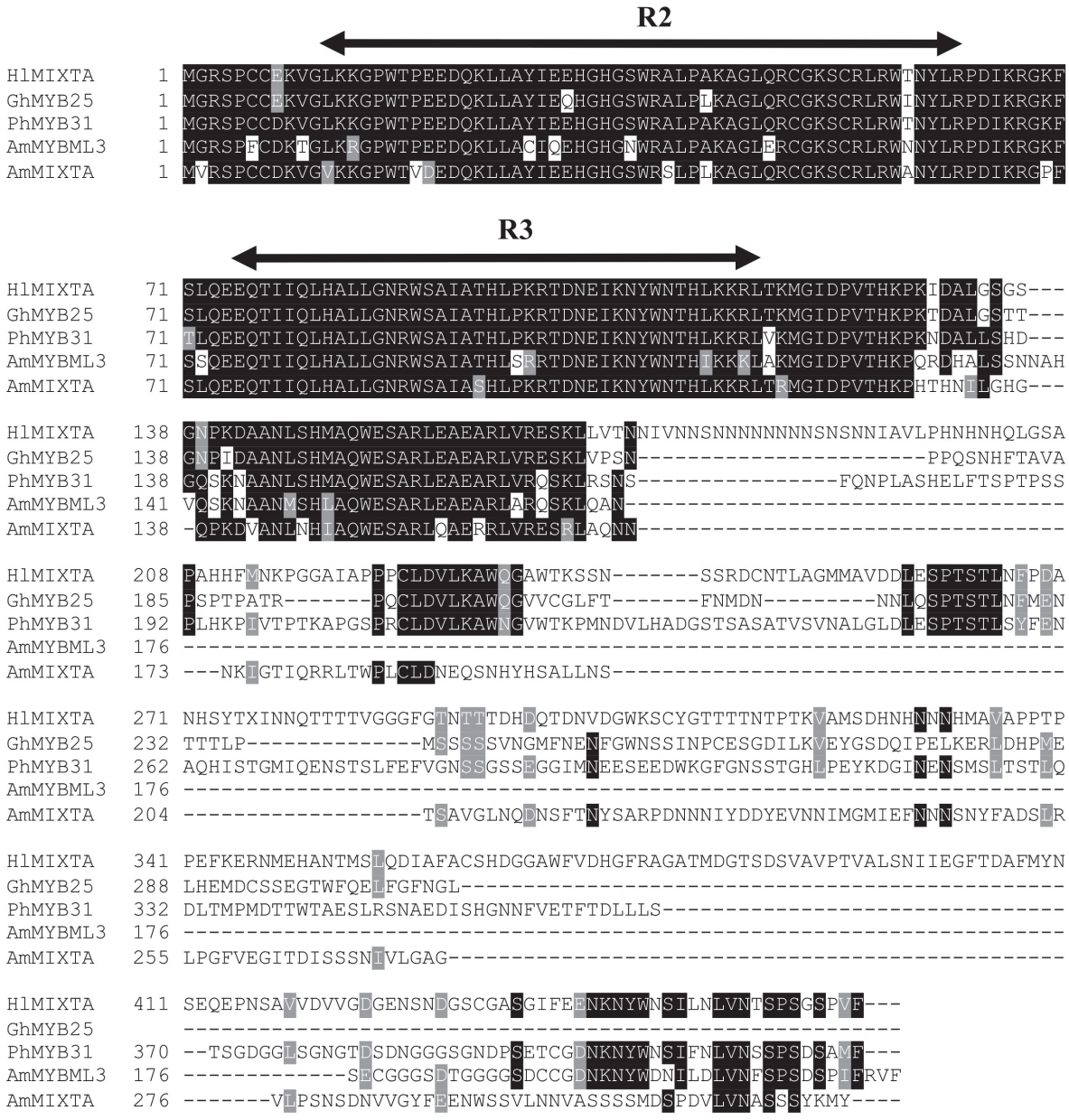

Figure 2. Multiple alignment of deduced amino acid sequence of *HlMIXTA1*.

Sequences were aligned using Clustal W, and shading was performed using Boxshade version 3.21. Identical amino acids are boxed in black; similar amino acids are boxed in gray. Predicted R2R3 domains are shown on the *HlMIXTA1* amino acid sequence by arrows. Accession numbers: GhMYB25 (AAK19616), PhMYB31 (CAA78386), AmMYBML3 (DQ228865), AmMIXTA (CAA55725).

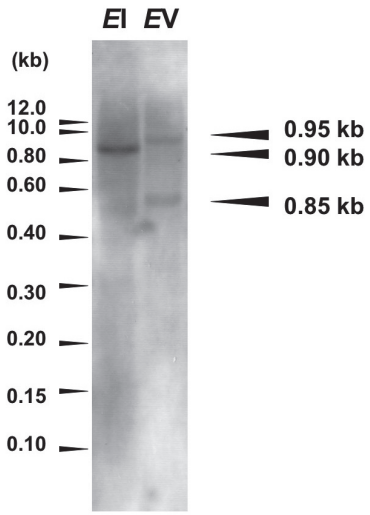

Figure 3. Southern blot analysis of *HlMIXTA1* gene.
Genomic DNA was digested with *Eco*RI (EI) and *Eco*RV (EV) and hybridized with a probe for the gene-specific region that does not have a *Eco*RI and *Eco*RV digested site. The size of the fragment is shown on the right.

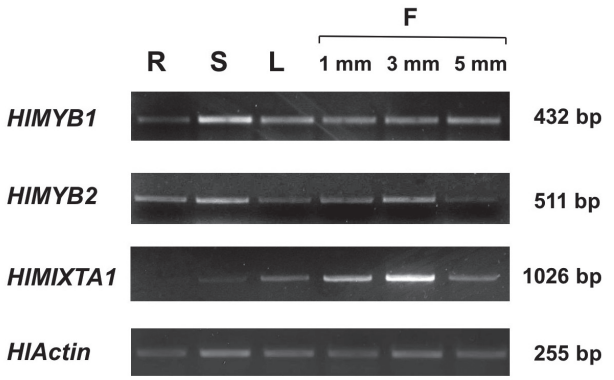

Figure 4. RT-PCR amplification of *HlMYB1*, *HlMYB2* and *HlMIXTA1*.
Poly(A)+ RNAs were isolated and reverse-transcribed from roots (R), stems (S), leaves (L), and female flower buds (1 mm, 3 mm, 5 mm) (F). *HlActin* gene was amplified as a control. The size of each PCR product is indicated on the right.

Tissue-specific expression patterns of *HlMIXTA1*

The expression patterns of the three isolated genes were tested in roots, stems, leaves, and female flower buds by RT-PCR (Fig. 4). *HlMYB1* was strongly expressed in stems. However, *HlMYB1* and *HlMYB2* were expressed in all RNA samples and did not show tissue-specific expression patterns. *HlMIXTA1* was mainly expressed in leaves and flower buds, showing weak expression in stems. These results were identified by glandular trichome distribution in hop plant tissues. Glandular trichomes develop in stems, leaves, and flowers. In these tissues, glandular trichomes are distributed in high density in young flowers. High accumulation of *HlMIXTA1* mRNA in flower buds indicates that this gene has a role in early development stage of glandular trichomes. Morphological observation with microscopy showed that glandular trichomes initiate from epidermal cells and in 3-mm flower bud.

Spatial expression of *HlMIXTA1* in young flowers

RT-PCR analysis indicates that *HlMIXTA1* is considered a candidate gene that regulates the initial stage of glandular trichome development. To examine detailed expression patterns, ISH was performed using 3-mm flower buds. According to DNA sequence homology search and Southern blot analysis, the gene-specific region of *HlMIXTA1* was isolated (Fig. 3) and used as a probe for ISH. A strong signal was detected in conical cells that become trichomes (Fig. 5B, closed arrowheads). Brown colored cells are not signals of gene expression by ISH (Fig. 5B). These cells accumulated condensed tannins. After reaction procedures for signal detection in ISH, tannins are oxidated and changed color to brown. Using the sense probe as a negative control, there were no signals in tissues, but brown cells were observed after ISH detection reaction (data not shown). These data suggest that *HlMIXTA1* works in the early developmental stage of trichome initiation from epidermal cells.

Discussion

To isolate regulatory genes for lupulin gland (glandular trichome) initiation in hop, RT-PCR was performed using degenerated primers designed from the conserved region of the MYB gene family. Three MYB-like genes, *HlMYB1*, *HlMYB2*, and *HlMIXTA1*, were isolated from hop female flowers. *HlMYB1* and *HlMYB2* expression was not specific for trichome development and was found in roots, stems, leaves, and flowers. *PbMYB3* (GenBank accession: KT601124), which has a highly similar MYB domain sequence with the *HlMYB1* gene, is reported to indirectly regulate flavonoid biosynthesis in pear fruit[9]. The same gene with *HlMYB2* was submitted to GenBank (accession: FN646081), and its function is unclear. Our ISH results show that *HlMYB1* and *HlMYB2* signals were located on tannin-accumulated cells (data not shown). This indicated

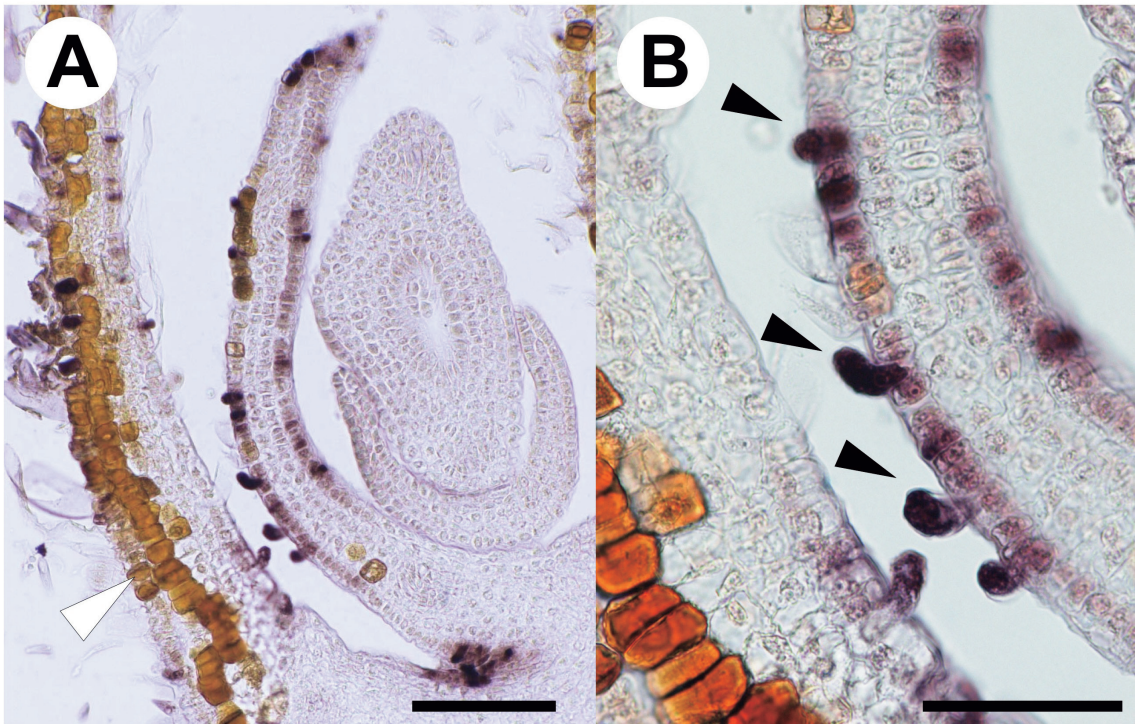

Figure 5. *In situ* hybridization of *HlMIXTA1* gene.

A. Longitudinal section of 3 mm size of female flower bud. B. Higher magnification of the bracteole from Fig. 5A. Closed arrowheads: *HlMIXITA1* expression in conical cells. Open arrowheads: brown colored cells that contain condensed tannin. Scale bars: 100 μm (A), 50 μm (B).

that these genes (*HlMYB1* and *HlMYB2*), or genes that have similar MYB domain sequences, regulate flavonoid biosynthesis. Meanwhile, *HlMIXTA1* was expressed in stems, leaves, and flowers but not in roots. ISH analysis using the 3' UTR region of *HlMIXTA1* as a probe showed clear signals on conical cells on the outer surface of the bracteoles of hop female flowers (Fig. 5). This suggests that *HlMIXTA1* might have some function in the early developmental stage of trichome initiation.

The developmental process of trichome has been well studied using the model plant, *A. thaliana*. The development is controlled by three major groups of transcription factors: the R2R3 MYB, bHLH, and WDR protein[10]. Thus, to induce additional glandular trichomes with genetic engineering, it is necessary to isolate other transcription factors and analyze their function. In this research, *HlMIXTA1* showed specific expression patterns on epidermal cells that have outgrowths to develop the trichomes. Fig. 5B shows the clear signals in epidermal conical cells. Glandular trichomes are distributed on the abaxial surface of bracteoles[3]. *HlMIXTA1* signals were detected mainly on the abaxial surface but also on the adaxial surface of bracteoles. Because hop female flowers also have non-glandular trichomes (hairs), it is not clear that these cells showing strong *HlMIXTA1* signals will develop into glandular trichomes or non-glandular trichomes or both.

MIXTA was first isolated from *Antirrhinum majus*[11]. Expression of *AmMIXTA* throughout transgenic tobacco plants leads to excess numbers of multicellular trichomes on leaves and floral organs as well as the novel production of conical cells on leaves[12]. Recently Shi et al. isolated *AaMIXTA1* from *Artemisia annua*[13]. Overexpression and repression of *AaMIXTA1* resulted in an increase in the number of glandular trichomes as well as the artemisinin content in transgenic plants. Here we reported on the MIXTA-like gene (*HlMIXTA1*) isolated from hop female flower that will play an essential role in the initiation of trichome development. Future studies will need to generate a *HlMIXTA1*-knockout plant to further investigate the regulatory mechanisms of glandular trichome initiation in hop.

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