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Development Note

Two distinct phases of glandular trichome development in hop (*Humulus lupulus* L.)

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Abstract Hop bitter resins and essential oils are biosynthesized and accumulated in lupulin glands of hop (*Humulus lupulus* L.). Valerophenone synthase (VPS) is involved in the first steps of biosynthesis of bitter resins, and the *VPS* gene is specifically expressed in lupulin glands on bracteoles and leaves. In this study, spatial and temporal expression of the *VPS* gene was studied using *in-situ* hybridization. No gene signals were observed in the early stage of lupulin gland development, but strong expression was observed when the cuticle was slightly detached from the glandular head cells. Expression levels were remarkably reduced with accumulation of hop bitter resins and essential oils in the sub-cuticular space. The findings suggest that development of lupulin glands is strictly divided into a growth phase and biosynthetic-secretory phase.

Key words: Glandular trichome, hop, *in-situ* hybridization, lupulin gland, valerophenone synthase.

Hop (*Humulus lupulus* L.) is an important material for beer brewing. Hop bitter resins and essential oils, which give beer its bitter taste and aroma, accumulate in glandular trichomes. Both the metabolic pathways and morphological features of hop glandular trichomes have been studied. For example, Roberts and Stevens (1962) documented the biosynthetic pathway of the essential oils while Mori (1961) documented resin biosynthesis. Moreover, Oliveira and Pais (1988) reported morphological changes in glandular trichomes with development.

Glandular trichome initiate by hypertrophy and anticlinal division of a protodermal cell, followed by establishment of glandular head cells. Three types of glandular trichome develop in hop plants, two peltate and one bulbous type. The peltate-type is known as lupulin glands, and are found on the abaxial side of bracteoles and leaves. Those found on the bracteoles of female flowers are used as the raw material in beer brewing. Lupulin glands consist of four basal cells, four stalk cells and a large flattened one-layered glandular head (Oliveira and Pais 1988). The glandular head consists of 100 to 200 cells (see Figure 2G). Those found on the leaves differ in size and shape from those on the bracteoles, consisting of 30 to 72 cells (see Figure 2N). Moreover, while those on the bracteoles are bicone in shape, those on the leaves are pancake shaped. Bulbous trichomes, on the other hand, are smaller and consist of four head cells as well as a small amount of secreted materials (see Figure 2S). As with the lupulin glands, bulbous trichomes mainly exist on the bracteoles and leaves.

Although lupulin glands increase in size with flower bud and leaf development (Oliveira and Pais 1988, Hirosawa et al. 1995), the details of this relationship are not clear. However, Oliveira and Pais (1988) revealed that the density of the lupulin glands on leaves decreased with leaf expansion, indicating that the final number of trichomes is established early in leaf development.

The timing of resin and essential oil biosynthesis is thought to be related to the developmental stages of glandular trichomes; however, no study has yet examined in detail the spatial and temporal expression of phytochemical biosynthetic genes during glandular trichome development. Okada et al. (2001) isolated the valerophenone synthase (VPS) gene, which is responsible for biosynthesis of the precursors of bitter resins, α-acid (humulone and cohumulone) and β-acid (lupulone and colupulone) (Figure 1), and specifically expressed in lupulin glands of hop. We therefore studied the spatial and temporal expression of *VPS* during the developmental stages of lupulin glands using *in-situ* hybridization.

In-situ hybridization was performed as in the reports of Ishii et al. (1997) and Sugiyama et al. (2006). Female flower buds were fixed according to their size (1 mm, 3 mm, 5 mm, 1 cm, and 3 cm), and large buds (1 cm and 3

Abbreviations: GUS, beta-glucuronidase; VPS, valerophenone synthase.
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cm) were divided into pieces of bracteole. Flower buds and young leaves (2.5 to 3 cm) were fixed in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde, dehydrated by a graded ethanol series (50 to 100%), embedded in Histosec (Merck, Darmstadi, German), and sliced into 12-μm sections. An exon 2-specific DNA fragment (460 bp) of the *VPS* gene was used as a probe. Two other *VPS* homologous chalcone synthase genes (*CHS4* and *CHS_H1*) are also expressed in lupulin glands (Okada et al. 2004), and thus, to avoid cross hybridization, hybridization and washing were performed at 65°C (Ishii et al. 1997, Novák et al. 2003). NBT/BCIP (4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) color detection was used, with a violet-purple color indicating the hybridizing signals (closed arrowheads in Figure 2). Cells in which tannins accumulated turned brown as a result of oxidation during hybridization (open arrowheads in Figure 2); tannins mainly accumulated in epidermal cells on the abaxial side of bracteoles.

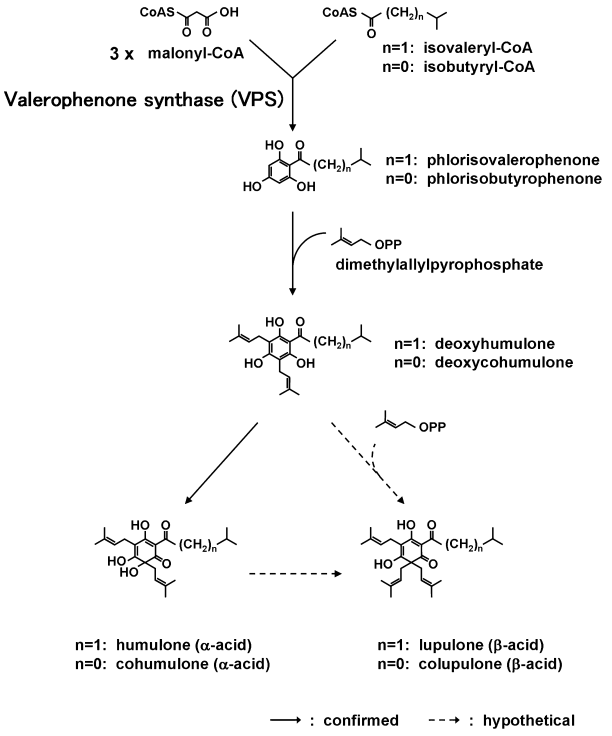

Figure 1. Schematic representation of the biosynthesis of bitter acids in hop plants. Adopted from Zuurbier et al. 1998 with modification.

Several developmental stages of lupulin glands were observed in the bracteoles. Although the development of each lupulin glands was not synchronized, they were shown to increase in size with flower bud development (Figure 2A–E). Three stages of development were defined based on morphological features of the lupulin glands on the bracteoles: Stage 1, from initiation to the formation of four glandular head cells (Figure 2A); Stage 2, growth of the glandular head cells from four to about 200 cells (Figure 2B); and Stage 3, the accumulation of secreted materials (Figure 2C, D, E).

In-situ hybridization analysis of the lupulin glands on the bracteoles demonstrated that *VPS* transcripts were not detected at stages 1 and 2 (Figure 2A, B). Subsequently, expression of the *VPS* gene started at stage 3 (closed arrowhead in Figure 2C), with signals showing a patchy distribution in the glandular heads (Figure 2C)

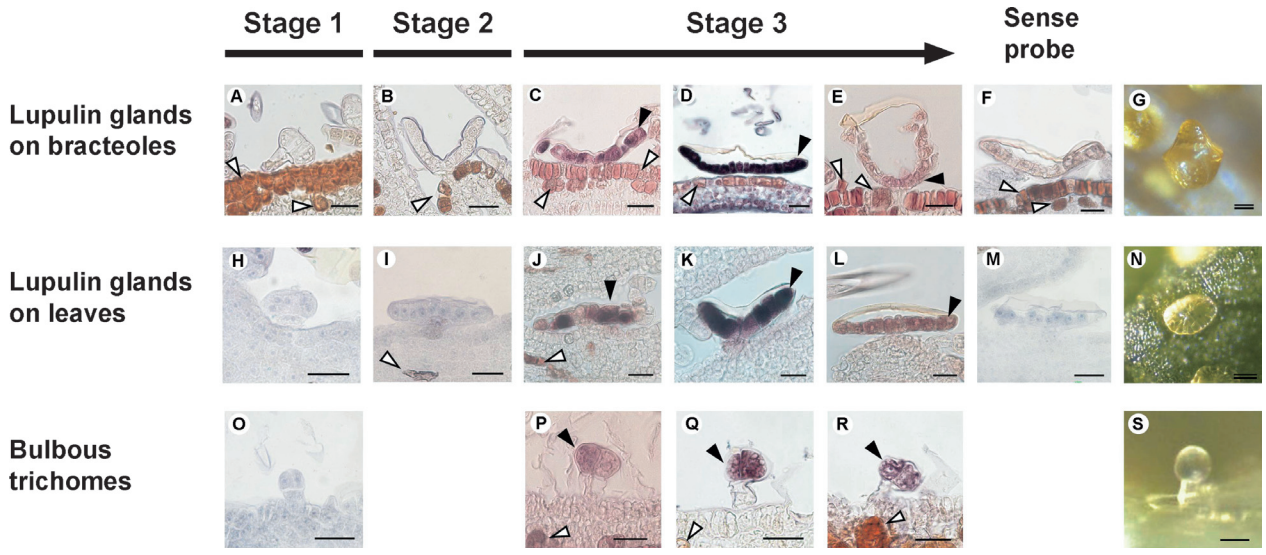

Figure 2. Expression patterns of the *VPS* gene in three types of glandular trichome. Stage 1 development of lupulin glands on a bracteole (A) and leaf (H). Brown-colored cells represent accumulation of tannins (open arrowheads). Stage 2 development of lupulin glands on a bracteole (B) and leaf (I). Stage 3 development of lupulin glands on a bracteole (C, D, E) and leaf (J, K, L). Purple-colored cells represent expression of the *VPS* gene (closed arrowheads). Hybridization with a sense probe at stage 3 development of lupulin glands on a bracteole (F) and leaf (M). Stage 1 development of bulbous trichomes (O). Stage 3 development of bulbous trichomes (P, Q, R). Mature lupulin glands on a bracteole (G) and leaf (N). A mature bulbous trichome on the adaxial side of a bracteole (S). Scale bars: 25 μm (A–F, H–M, O–S), 50 μm (G, N).

when the cuticle was slightly detached from the glandular head cells. Expression levels of the *VPS* gene rapidly increased (Figure 2D); however, as soon as accumulation of secreted materials increased, expression levels once again reduced (Figure 2E).

Young leaves (2.5 to 3 cm) were also used for *in-situ* hybridization, revealing a high density and several developmental stages of lupulin glands. Expression patterns of the *VPS* gene in the lupulin glands on the leaves were the same as those on the bracteoles. No expression was detected at stages 1 and 2 (Figure 2H, I), but started to appear at stage 3 (Figure 2J, K). Weak signals were observed after accumulation of secreted materials (Figure 2L).

Signals of the *VPS* gene were also detected in bulbous trichomes. During early stages of glandular trichome development (stage 1), four-head-celled trichomes could not be identified as bulbous trichomes (Figure 2P, Q, R) or lupulin glands (Figure 2A, H) by their morphological features. Glandular trichomes on the adaxial side of the bracteoles were therefore considered to be bulbous glandular trichomes because few lupulin glandular trichomes are found here. No tannin accumulation was observed in epidermal cells on the adaxial side of the bracteoles (Figure 2O, P, Q, R).

Mature bulbous trichomes have only four glandular head cells. Compared to the above definition of the developmental stages for lupulin glands, bulbous trichomes did not show stage 2. Moreover, stage 3 could not be identified because only a small amount of secreted materials accumulates temporarily in bulbous trichomes. We therefore classified putative stages based on morphological features and expression patterns of the *VPS* gene. Signals of the *VPS* gene were detected in some four-head-celled bulbous trichomes, and expression levels differed among head cells (Figure 2P). This expression pattern was the same as that of the lupulin glands at stage 3 (Figure 2J). The bulbous trichomes shown in Figures 2Q and 2R are therefore thought to represent the same developmental stages as the lupulin glands in Figures 2K and 2L, respectively.

Based on our *in-situ* hybridization data, development of both lupulin glands and bulbous trichomes was shown to have two distinct phases: a growth phase (stage 1 to 2) and biosynthetic-secretory phase (stage 3). Identical developmental features have also been reported for glandular trichomes of mint (Turner et al. 2000) and three Finnish birches (Valkama et al. 2004), which have a one-layered glandular head. However, these studies were based on morphological features only. Turner et al. (2000) and Valkama et al. (2004) showed that glandular trichomes function mainly as storage organs in the post-secretory phase. We also suggest that glandular trichomes with a one-layered glandular head have three developmental phases: a growth phase, biosynthetic-secretory phase and storage phase.

Okada et al. (2003) analyzed the promoter region of the *VPS* gene using a glucuronidase *uidA* gene (beta-glucuronidase; GUS) as the reporter gene, revealing GUS activity only in the central region of the glandular head. In our *in-situ* hybridization, signals of the *VPS* gene were detected in all cells of the glandular head (Figure 2D, K). Secreted materials start to accumulate from the edge of the flattened one-layered glandular head (Hirosawa et al. 1995). Oily metabolites in the secreted materials might therefore prevent the substance in the GUS assay from soaking into cells on the edge of the glandular head. Reporter gene assays, which require extracellular substances, are therefore not suitable for analysis of accumulated oily metabolites in plant cells.

Okada et al. (2003) also showed that mature lupulin glands do not stain, while ruptured lupulin glands do. This result indicates that the *VPS* gene might be expressed in mature lupulin glands. Our *in-situ* hybridization data showed that signals of the *VPS* gene were reduced in mature lupulin glands after accumulation of secreted materials (Figure 2E). This could be because of differences between the lifetime of the GUS protein and that of the *VPS* transcripts.

The GUS assay conducted by Okada et al. (2003) did not show *VPS* promoter activity in bulbous trichomes, unlike in our *in-situ* hybridization. Signals of the *VPS* gene in bulbous trichomes (Figure 2Q) were lower than those in lupulin glands (Figure 2D, K), suggesting that the level of *VPS* gene expression in bulbous trichomes might be insufficient for detection in the GUS assay.

In conclusion, we revealed the detailed spatial and temporal expression patterns of the *VPS* gene during the developmental stages of glandular trichomes using *in-situ* hybridization. *In-situ* hybridization is a suitable method for studying activation of a promoter based on spatial and temporal expression patterns of a target gene, because it does not require transgenic plants and is able to detect even weak levels of gene expression.

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