Characteristic Thickened Cell Walls of the Bracts of the ‘Eternal Flower’

Helichrysum bracteatum

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INTRODUCTION

Helichrysum bracteatum has compound flowers comprised of many tubular flowers and scarious bracts (Everett, 1980). The scarious bracts are large and coloured like a corolla. They maintain their aesthetic value without wilting or discoloration for many years, even after cutting. Helichrysum bracteatum is, therefore, suitable as a dried flower. Many species in the Compositae family have compound flowers comprised of many tubular flowers and scarious bracts (Everett, 1980). The scarious bracts of H. bracteatum ‘Jumbo Yellow’ was 38.6 %, and that of the scarious sepals of L. sunuatum ‘Sundaeviolet’ was 21.2 %. Water contents of the leaves of these species were 88.9 %, 91.0 % and 75.9 %, respectively. Thus, scarious tissues have a low water content, while growing plant tissues typically contain 80 to 90 % water. Wood is composed mostly of dead cells, and has a low water content. For instance, the sapwood that functions in transport in via the xylem contains 35 – 75 % water (Taiz and Zeiger, 2002). The scarious bract of C. acaulis is composed of dead cells (Troll, 1957). These observations suggest that H. bracteatum scarious bracts and L. sunuatum scarious sepals are composed of dead cells. However, to the best of our knowledge, no research studies show this, nor are there reports of the cell morphology of scarious bracts and sepals.

We investigated whether the cells of the scarious bracts of H. bracteatum ‘Monstrosa’ are dead or alive by observing nuclei of such cells stained by DAPI (4’6-diamidino-2-phenylindol dihydrochloride) under a fluorescence microscope, and examined the morphology of scarious bract cells under a light microscope, a transmission electron microscope and a polarized light microscope.

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MATERIALS AND METHODS
Plants of Helichrysum bracteatum ‘Monstrosa’ were cultivated in a plastic greenhouse at Kyoto University in Japan. They were grown in pots containing the growing medium Metro-Mix 360 (Sun Gro Horticulture Canada Ltd, Seba Beach, Canada) under natural sunlight. The composition of this medium is peat moss, vermiculite, bark ash, bark, dolomitic limestone and a wetting agent. The solid fertilizer IBS1 (N : P : K = 1 : 1 : 1; JA Group, Tokyo, Japan) was applied. Flowers of these plant were used in the following experiments.

DAPI staining and fluorescence microscopy
Seven stages of H. bracteatum flower development were considered (Fig. 1A): stage 1, bud <8.0 mm wide; stage 2, bud 8.0–10 mm wide; stage 3, bud 10–12 mm wide; stage 4, bud 12–14 mm wide, with its second layers of bracts starting to open; stage 5: 4th–5th bracts of the bud starting to open; and stage 7, all bracts completely opened (anthesis). The innermost bracts at each stage of flower development, or bracts adjacent to tubular flowers, were used in the following experiments, which were performed according to Gladish et al. (2006). Bracts at each stage were stained with 1 mg L⁻¹ DAPI (4’6-diamidino-2-phenylindol dihydrochloride) in 10 mmol L⁻¹ Tris/HCl buffer (pH 7.4). The bracts were soaked in DAPI solution in a vacuum pump in the dark overnight to completely stain the nuclei of all cells of the bracts. Nuclei of the bracts were observed using a fluorescence microscope (Olympus BX60). Ultra-thin sections were cut using an ultramicrotome (Reichert-Jung). These were stained with aqueous uranyl acetate and Reynold’s lead citrate and observed under a transmission electron microscope (JOEL JEM-1220).

Polarized light microscopy
A bract of H. bracteatum ‘Monstrosa’ at stage 7 (anthesis) was used. A petal and a bract of Chrysanthemum morifolium ‘Piato’ were also used for comparison. A razor blade was used to hand-section 3-mm wide segments of each tissue, which were fixed in FAA [100 % ethanol : DW : formalin : acetic acid = 12 : 6 : 1 : 1 (v/v)] overnight at room temperature, and dehydrated through a graded ethanol series: 30 %, 50 %, 70 %, 80 %, 90 %, 95 %, 95 %, 95 %, 99.5 % and 99.5 % for 60 min each. They were embedded in Technovit 7100 resin (Heraeus Kuzer). Semi-thin sections were cut using a rotary microtome (Leica RM2155), and were observed under a light microscope (Olympus BX60). Ultra-thin sections were cut using an ultramicrotome (Reichert-Jung). These were stained with 2 % aqueous uranyl acetate and Reynold’s lead citrate and observed under a transmission electron microscope (JOEL JEM-1220).

RESULTS
Nuclei in cells of bracts at each stage
Fluorescence of many nuclei in individual cells was observed at the bract tip at stage 1 (Fig. 2A); fewer nuclei were observed at stages 2 and 3 (Fig. 2B, C) and most had disappeared at stage 4 (Fig. 2D). In contrast, fluorescence of many nuclei was observed in bract bases at all stages (data not shown). The ratio of nuclei to epidermal cells in the parts tip–1/4 and 1/4–2/4 decreased with stage advancement (Fig. 3). The ratio in the 1/4–2/4 part at stages 1 and 2 were over 1.0; the reason for this is that the number of nuclei was determined in both the underlying layers of the epidermis as well as in the epidermis itself, whereas the cell number was only counted in the latter. No nuclei were observed in the tip–1/4 part at stage 5; thus, cell death occurred at the bract tip at the...
early stage of flower development. Most nuclei disappeared in the upper half of the bract by stage 5 (before anthesis).

Cell morphology and characteristic secondary cell wall

Spongy parenchyma was observed in petal and bract tissues of *C. morifolium* (Fig. 4B, C), but not in *H. bracteatum* bract tissue (Fig. 4A). Cells of *H. bracteatum* were closely arranged and smaller than those of *C. morifolium*.

A large vacuole was observed at the centre of cells of the petal and bract tissues of *C. morifolium* (Fig. 4F–I), with cytoplasm surrounding the vacuole. Some organelles, such as the nucleus and chloroplasts, were observed in the cytoplasm. On the other hand, no organelles were observed in the cells of *H. bracteatum* bract tissue (Fig. 4D, E). The cell walls were the most prominent characteristic of cells of the *H. bracteatum* bract. The primary cell walls form the outermost layer of cells in all tissues of the two species. Only primary cell walls were observed in the cells of the petal and bract of *C. morifolium* (Fig. 4F–I), whereas characteristic thickened secondary cell walls on the inside of the primary walls were observed in both epidermal and inner cells of the *H. bracteatum* bract (Fig. 4D, E). The outer periclinal walls of all epidermal cells were thickened primary cell walls in petal and bract tissue of *C. morifolium* (Fig. 4D, E). On the other hand, there were two layers, comprised of a thin primary cell wall and a thickened secondary cell wall, in all epidermal cells in the bract tissue of *H. bracteatum* (Fig. 4D). A cuticle layer was observed on the outside of the primary cell walls in all the tissues of the two species that were examined. Only flat primary cell walls of all inner cells, except for tracheary elements, were observed in the petals and bracts of *C. morifolium* (Fig. 4G, I), whereas in bract tissue of *H. bracteatum*, secondary cell walls on the inside of flat primary cell walls of all the inner cells had irregular thickening as lobes (Fig. 4E).

Birefringent properties of cell walls

The general secondary cell wall in tracheary elements (tracheid and vessel) or fibres has a highly birefringent property that can be observed under a polarized light microscope (Leney, 1981; Lev-Yadun, 1997; Jang, 1998; Bergander et al., 2002; Donaldson and Xu, 2005; Thygesen and Hoffmeyer, 2005; Lev-Yadun et al., 2005). This is because cellulose, which is the main component of the secondary cell wall, has crystalline properties resulting from the orderly arrangement of cellulose molecules in microfibrils (Smith et al., 1998). The orientation of cellulose microfibrils is neatly aligned parallel to each other in secondary cell walls (Taiz and Zeiger, 2002), so that birefringence is generated when viewed under polarized light (Evert, 2006). The primary cell wall cannot be observed under a polarized light microscope because it has a rather random arrangement of microfibrils.

Tracheary elements exhibited birefringence in petal and bract tissues of *C. morifolium* (Fig. 5E, H), because they have a secondary cell wall. The outer periclinal wall of epidermal cells also exhibited birefringence in *C. morifolium* bract tissue (Fig. 5H); although it is a primary cell wall, it consists of many layers (Fig. 4H) and so it exhibited birefringence. Observation under a polarized light microscope with a compensator showed the orientation of cellulose (Fig. 5C, F, I). In the figure, blue and yellow interference colours are vertical; blue interference colour shows that the orientation of cellulose is parallel to the x-axis, whilst yellow interference colour shows that the orientation of cellulose is parallel to the z-axis. Tracheary elements in petal and bract tissues of *C. morifolium* had two vertical orientations of cellulose (Fig. 5F, I). However, the cell walls of

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**Fig. 3.** Ratio of nuclei to epidermal cells in two parts (tip–1/4 and 1/4–2/4) of the *H. bracteatum* bract (see Fig. 1). Bars represent the mean value ± s.e. of three independent experiments.
parenchyma cells, except for those of tracheary elements and the outer periclinal walls, exhibited no birefringence in *C. morifolium* petal and bract tissues. On the other hand, cell walls of all the cells exhibited birefringence in *H. bracteatum* bract tissue (Fig. 5B). They had the same orientation of cellulose as tracheary elements in *C. morifolium* petal and bract tissues (Fig. 5C). Moreover, the orientation of cellulose of the outer periclinal walls was parallel to the *x*-axis, not parallel to the *z*-axis in the *H. bracteatum* bract (Fig. 5C).

**DISCUSSION**

Most nuclei disappeared in the upper half of the bract of *H. bracteatum* before anthesis, as observed by DAPI staining and fluorescence microscopy (Figs 2, 3); moreover, no organelles were observed in the cells of the *H. bracteatum* bract (Fig. 4D, E). For these reasons, it is shown that the bracts at anthesis are composed of dead cells. Characteristic thickened secondary cell walls on the inside of the primary cell wall were observed in all cells of the bract tissue of *H. bracteatum* by TEM (Fig. 4D, E).
The walls of all the cells of *H. bracteatum* bract tissue exhibited birefringence, as determined by polarized light microscopy (Fig. 5B, C); the birefringence arises from the characteristic thickened secondary cell wall. It is therefore suggested that these walls of *H. bracteatum* bract cells have orientated cellulose microfibrils, as do the secondary cell walls of the tracheary elements of petal and bract tissues of *C. morifolium*. The birefringence at the outer periclinal walls of the epidermal cells of *C. morifolium* bract tissue (Fig. 4H, I) may be from many layers of primary cell walls. In summary, the bract of *H. bracteatum* is composed of dead cells, which have characteristic thickened secondary cell walls. These secondary cell walls have orientated cellulose microfibrils.

Cell walls of plants are classified into two types, primary and secondary. Growing cells, which have a viscoelastic property and can expand, form primary cell walls. Secondary cell walls are formed on the inside of primary cell walls after cell growth has ceased. They function in mechanical support in plants. They contain a higher
proportion of cellulose than primary cell walls, and the orientation of cellulose microfibrils may be more neatly aligned parallel to each other than in primary cell walls (Taiz and Zeiger, 2002). Due to this property, only secondary cell walls exhibit birefringence. Moreover, cells with secondary cell walls are often dead. Are the characteristic secondary cell walls of the H. bracteatum bract the same as general secondary cell walls? We compared cells of the bract of H. bracteatum with taxonomical cells with general secondary cell walls.

Plant cells are classified into many cell types. According to the classification of Raven et al. (2005), cells with secondary cell walls are classified into three types: specialized parenchyma cells, sclerenchyma cells, and cells of tracheary elements. Sclerenchyma consists of fibres and sclereids, whilst tracheary elements consist of vessels and tracheids.

Leaves and petals contain a high amount water, and are composed mostly of parenchyma cells with primary cell walls and living cytoplasm. The cells of petal and bract tissues of C. morifolium had only a primary cell wall (Fig. 4F–I) and no birefringent properties (Fig. 5E, F, H, I). ‘Transfer cells’ are specialized parenchyma cells with secondary cell walls. Morphologically two categories of cell wall ingrowths can be recognized for most transfer cells; reticulate and flange (Pate and Gunning, 1972; Talbot et al., 2002). The shape of the secondary cell walls of the H. bracteatum bract is similar to that of transfer cells; however, cells of the H. bracteatum bract differ from transfer cells in their function, their non-living state and their location. Lobes of the secondary cell walls of transfer cells are for the transfer of solutes over short distances (Gunning and Pate, 1969; Gunning, 1977; McDonald et al., 1996; Harrington et al., 1997), and hence these cells are not dead. The locations of transfer cells are potential sites of intensive short-distance solute transfer, for example xylem, phloeum and tissues of reproductive and glandular structures (Gunning et al., 1970; Rost and Lersten, 1970; Diane et al., 2002; Pate and Gunning, 1972).

Cells of the H. bracteatum bract are similar to sclerenchyma cells in function. The principal function of sclerenchyma cells is mechanical support, and these cells have secondary cell walls. However, cells of the H. bracteatum bract differ from fibres, which are a kind of sclerenchyma cell, in the location and the shape of secondary cell walls. The locations of fibres are the xylem, phloeum, hypodermis, cortex and central cylinder (Evert, 2006), and secondary cell walls of fibre cells form a flat, thickened layer (Evert, 2006). Another type of sclerenchyma cell, sclereids, also have various types; leaves are a rich source of sclereids (Foster, 1955, 1956; Esau, 1977; Karabourniotis et al., 1994; Karabourniotis, 1998). Cells of the H. bracteatum bract also differ from sclereids in their overall shape, the shape of the secondary cell walls, their non-living state and their location. The sclereids of leaves have numerous simple pits, are often alive at maturity, and are located in particular parts of leaves, for example the ends of small veins, patches, epidermis and intercellular spaces.

Cells of the H. bracteatum bract are similar to those of tracheary elements in their non-living state and the shape of the secondary cell walls. Cells of tracheary elements are dead at maturity and their secondary cell walls have irregular thickness and form some lobes (Esau and Charvat, 1978; Burgess and Linstead, 1984; Groover et al., 1997; Karlsson et al., 2005). However, cells of the H. bracteatum bract differ from tracheary elements in their function; the principal function of tracheary elements is in water conduction.

In conclusion, cells of the H. bracteatum bract differ from those of other tissues whose characterized cells have secondary cell walls, and they may be a new cell type. All cells of the H. bracteatum bract have characteristic thickened secondary cell walls that have not been reported in the parenchyma of any other flowers or leaves. Cells of the H. bracteatum bract are therefore an interesting subject for further research on differentiation and development.

LITERATURE CITED


