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Histochemical study of the development of the phytomelan layer in the seed coat of *Gasteria verrucosa* (Mill.) H. Duval

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Summary. In this study we document the development of the phytomelan layer in the outer epidermis of the outer integument of *Gasteria verrucosa*. Phytomelan has been described as a black, melanin-like substance which is chemically very inert. Using histochemical techniques we show that phytomelan development in the cell wall can be divided into three stages. The first stage is deposition of a callosic layer against the tangential wall, with simultaneous thickening of the adjacent parts of the radial walls. The second stage is the conversion of this callosic wall, which we call a tertiary wall, into a non-callosic inner and outer layer. The inner layer stains predominantly for cellulose and a little for pectin. The outer layer is of unknown composition, since it did not react with the stains that were used. In the third stage the outer tertiary layer becomes black, the phytomelan. The callosic wall deposited in the first developmental stage seems to function as a carbohydrate source and as a mould for the tertiary cell wall. The conversion of the callose in the second stage might be the result of penetration of substances which react with callose. All the components for phytomelan seem to be present in the outer layer before the conversion. Phenolics might be involved in this second conversion.

Keywords: Callose; Cell wall; *Gasteria*; Melanin; Phytomelan layer; Seed coat.

Abbreviations: DAP days after pollination; PAS periodic acid-Schiff's reagent; PEG polyethylene glycol.

Introduction

The ovule of *Gasteria verrucosa* (Mill.) H. Duval (Asphodelaceae, subfamily Alooideae) is anatropous and has a two cell thick inner integument and a three cell thick outer integument. Like most Asphodelaceae

(Huber 1969) *Gasteria* develops a phytomelan layer in the outer epidermis of the outer integument. After formation of the phytomelan layer the cytoplasm of the other integumental cell layers degenerates. The remaining cell walls become slightly compressed. A funicular arillus envelops the lower half of the mature ovule and expands after fertilization into a wing shaped structure enclosing the entire ovule. This arillus is generally four cells thick and dehydrated in mature seeds.

In the monocotyledons phytomelan seem to be most common in (and perhaps restricted to) the seeds of the order Asparagales (Dahlgren and Clifford 1982). Development of a phytomelan layer is shown in drawings of *Asparagus officinalis* by Robbins and Borthwick (1925) and of *Muscari commosum* and *M. racemosum* by Wunderlich (1937). In *A. officinalis* the phytomelan layer seems to comprise epidermal cells containing phytomelan deposits, while in *M. commosum* and *M. racemosum* it seems to be a continuous layer, covering the epidermal cells like a cuticle.

Takhtajan (1985) characterized the seed coat of the order Asparagales as a melanin cork: a black pigment formed by melanization, phytomelanin, that is stored in cells. Dahlgren and Clifford (1982) described phytomelan as opaque, brittle and charcoal-like. It is known that phytomelan is chemically very inert and has a carbon : hydrogen : oxygen ratio of 3.7 : 2.1 : 1.0 (Dafert and Miklausz 1912), we know of no chemical study of phytomelan that characterizes it

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as a type of melanin. In general, melanins are high molecular weight compounds formed by enzymatic oxidation of phenolics. They are difficult to study because they are resistant to chemical degradation (Thomson 1976).

Possible functions of the phytomelan layer in the seed coat are protection against fungal and microbial invasion, and prevention of deterioration, dehydration and radiation (Netolitzky 1926, Dahlgren and Rasmussen 1983). According to Takhtajan (1985) the phytomelan layer has a mechanical function in the seed coat. The phytomelan layer is used in plant systematics as an identification character. It has never been studied well enough to know what phytomelan is, how it is deposited in the seed coat, and whether it is of the same composition and structure in all the plant families which contain it. In this study we use histochemical techniques to investigate the development of the phytomelan layer in the seed coat of *Gasteria*.

Materials and methods

Plants of a self-incompatible hybrid population of *Gasteria verrucosa* (Mill.) H. Duval were grown in the greenhouse at 16 h light periods between 18 and 25 °C. Flowers were cross-pollinated in November and December 1993. Seeds were collected every day from 14 days after pollination (DAP) until 20 DAP and then 22, 25, 29, 38, and 45 DAP (mature seed).

The seeds were hand sectioned into 1 mm thick slices and fixed for 45 min in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at room temperature. After dehydration in an alcohol series the material was embedded in Technovit 7100 (Kulzer), in polyethylene glycol (PEG) (Merck), or, after dehydration through a tertiary butyl alcohol series in paraplast (Brunschwig Chemie). Technovit sections (7 µm) were used directly for staining. PEG sections (5 µm) were transferred with PEG 6000 (Merck) to poly-L-lysine (Sigma) coated slides and rinsed with water before staining. Paraplast sections (5 µm) were deparafinized through a xylol, tertiary butyl alcohol, alcohol and water series. Fresh seeds were embedded in Tissue-Tek (Miles), frozen at -20 °C and sectioned in a Micron CR 50-H Bio-Med cryostat. The sections were stained for (a) callose with 1.5% resorcin blue in 0.5% NH₄OH or 0.005% aniline blue (Eschrich and Currier 1964), (b) cellulose with zinc chlor-iodide (Jensen 1962) or 1% congo red (Locquin and Langeron 1983), (c) pectin with 0.1% ruthenium red (Jensen 1962), (d) lignin with saturated phloroglucinol in 20% HCl (Jensen 1962), (e) lignin and some polyphenols which stain green with the polychromatic stain toluidine blue (0.025%, pH 4.0) (Feder and Wolf 1965, O'Brien et al. 1965), (f) carbohydrates with periodic acid-Schiff's reagent (PAS) (Jensen 1962), omitting the oxidation reaction with periodic acid for controls, (g) proteins with 1% amido black in 7% acetic acid (Schneider 1981), and (h) cutin, suberin and fats with saturated Sudan IV in 95% ethanol (Schneider 1981). The cellulose stains and the ruthenium red may also stain some hemicelluloses and oxidized cellulose (Jensen 1962, Frey-Wyssling 1976), while a negative reaction does not necessarily mean that the component being stained for is absent. It may have been extracted during embedding. It is also possible that the stain cannot reach binding

sites because they are covered with other wall material, or that large numbers of binding sites must be present for staining to be detected (Jensen 1962).

The sections were photographed using bright field, polarizing light, differential interference contrast, or UV microscopy.

In order to remove the wall matrix paraplast sections of 22 DAP seeds were mounted on glass slides coated with Kaiser's glycerol gelatin (Merck). They were then deparafinized, and boiled for 15 or 45 min in a mixture of 30% hydrogen peroxide and glacial acetic acid (1 : 1 v/v). When the material has been treated long enough only cellulose microfibrils remain (Willemse 1972, Desphande 1976, Emons 1988). The treated sections were stained for cellulose with zinc chlor-iodide.

Results

Between 14 and 25 DAP wall material is deposited against the existing secondary wall of the outer epidermal cells of the outer integument. Because this material differs from that of the secondary walls, we use the term "tertiary wall". The formation of phytomelan in this tertiary wall results in a black epidermal layer enveloping the developing ovule (Fig. 1), except at the micropylar opening and the hilum.

Based on morphological changes we distinguish three developmental stages. The first stage (14–16 DAP) is the thickening of the radial secondary walls and the deposition of a tertiary wall against the outer secondary tangential wall (Fig. 2 A). The second stage (17–19 DAP) is the conversion of the tertiary wall (Fig. 2 B) into a two layered structure, an inner and an outer (Fig. 2 C). The inner layer is PAS positive while the outer layer is PAS negative. In the third stage (20–25 DAP) material of the outer layer is converted into phytomelan (Fig. 2 D).

Fig. 1. A 20 µm cryosection of a mature seed. The phytomelan layer (arrowhead) envelops the ovule. Openings in the phytomelan layer at the micropyle (*mi*) and hilum (*hi*) are not visible. An arillus (*ar*) covers the integuments. *em* Embryo; bar: 500 µm. Bright field

Stage 1 (14–16 DAP): deposition of the tertiary wall

During the first developmental stage the radial secondary walls thicken and the tangential tertiary wall is deposited. Radial secondary wall thickening starts at the outer tangential side of the wall. About two thirds of the wall thickens and can be stained for cellulose (Fig. 3 A), but not for pectin or lignin. Staining for pectin develops in the radial secondary wall when the thickening is completed, initially on the tangential side (Fig. 3 B, C). A tertiary wall is deposited against the outer tangential secondary wall, between the thickened radial walls. The callose stains aniline blue and resorcin blue both stain this tertiary wall (Fig. 3 D). PAS also produces a positive reaction (Fig. 2 A) but the PAS control and stain for cellulose, pectin, lignin, phenolics, proteins, and fatty substances do not react.

Stage 2 (17–19 DAP): first conversion of the tertiary wall

In the second stage the callose wall is transformed (Fig. 4 A) into two non-callosic layers. Just before this occurs proteins in the inner tangential layer of the callose wall, against the plasma membrane, become stainable. Protein staining is more distinct when the callose is being modified (Fig. 4 B). After conversion of the callosic tertiary wall the inner tertiary layer can be stained for cellulose (Fig. 4 C, D), slightly for pectin, but no longer for callose or proteins. The border between the inner and outer layer is not very distinct (Fig. 2 C) and the inner layer near the plasma mem-

brane shows isotropy of the cellulose with polarized light (Fig. 4 D). The conversion of the outer callosic layer starts at its inner tangential and radial borders, resulting in a distinctive pattern (Figs. 2 B and 4 A). After conversion the outer tertiary layer cannot be stained with cellulose, pectin, lignin, phenolic, carbohydrate (Fig. 2 C), protein, or lipid stains.

The secondary walls show no differences after conversion of the tertiary wall. At the end of the first conversion the cytoplasm of these epidermal cells degenerates leaving a lumen (Fig. 4 D).

Stage 3 (20–25 DAP): second conversion of the tertiary wall

In the third stage the outer layer of the tertiary wall gradually becomes brown (Figs. 2 D and 5 A–C) and eventually black (Fig. 5 D). The darkening outer tertiary layer cannot be stained for cellulose, pectin, lignin (by phloroglucinol), carbohydrates, proteins, or fatty substances. Only toluidine blue stains the darkening wall green (Fig. 5 A, C), indicating the presence of phenolics. The outer tertiary layer shows neither UV autofluorescence nor polarization effects. At about 25 DAP the outer tertiary layer is fully developed and black (Fig. 5 D), masking any staining reactions. The inner tertiary layer does not show any differences after the second stage.

During the treatment of 22 DAP seed sections with hydrogen peroxide-glacial acetic acid the dark brown outer tertiary wall layer pales and seems to disappear after 45 min treatment (Fig. 6). The persistent inner

Fig. 2. PAS stained Technovit sections. A–D, $\times 975$, bright field. **A** Stage 1: thickening of the radial secondary wall (*sw*) of the outer epidermal cells of the outer integument, and deposition of PAS positive tertiary wall (*tw*). *cy* Cytoplasm. **B** Stage 2: conversion of the tertiary wall material into PAS negative material (arrows). *ar* Arillus. **C** The tertiary wall after first conversion: an PAS positive inner layer (*il*) and a PAS negative outer layer (*ol*). **D** Stage 3: second conversion (darkening) of the outer tertiary layer. The inner tertiary layer and secondary walls are PAS positive. The outer tertiary layer is PAS negative and converts into phytomelan. *cl* Cell lumen

Fig. 3. Stage 1: deposition of the tertiary wall. A–C Paraplast sections and **D** PEG section. A–D, $\times 975$, differential interference contrast. **A** Zinc chlor-iodide stains the cellulosic radial secondary walls (*sw*) during deposition of the callosic tertiary wall (*tw*). **B** Local pectin staining (arrow) by ruthenium red in the radial secondary wall at the end of the deposition of the callosic tertiary wall. **C** Pectin staining of the entire radial secondary wall (*sw*) after callose deposition. **D** Resorcin stains the tertiary wall (*tw*) blue, indicating the presence of callose

Fig. 4. Stage 2: first conversion of the tertiary wall. **A** PEG section, **B–D** paraplast sections. A–D, $\times 975$, differential interference contrast (A and D) and bright field (B and C). **A** Resorcin blue staining shows callose patterns (*ca*) during the first conversion of the tertiary wall. **B** Amido black staining reveals proteins in the inner tertiary layer (*il*). *ol* Outer tertiary layer. **C** Zinc chlor-iodide stains the cellulose in the inner layer. **D** The end of the first conversion; the cellulose stain congo red in combination with polarised light showing cellulose fibrils in the secondary wall and cellulose isotropy in the tertiary inner layer. A cell lumen (*cl*) remains after degeneration of the cytoplasm

Fig. 5. Second conversion of the tertiary wall. **A–D** Paraplast sections, $\times 975$. **A** At the start of the second conversion the outer layer stains (*ol*) green with toluidine blue, indicating the presence of phenolics. *il* Inner layer, *cl* cell lumen. **B** Zinc chlor-iodide stains the cellulose of the inner layer. The outer layer is darkening. **C** Toluidine blue stains the brown outer layer green. **D** Zinc chlor-iodide stains the cellulosic inner layer (arrow). The outer layer is fully converted into phytomelan

Fig. 6. Sections (22 DAP) treated with hydrogen peroxide-glacial acetic acid. A–D, $\times 975$, differential interference contrast. **A** 0 min. Dark brown outer tertiary layer (*ol*), inner tertiary layer (*il*), and cell lumen (*cl*). **B** 15 min. The outer layer is partly dissolved. **C** 45 min. Persistent secondary walls (*sw*) and inner layer; the outer layer seems to be dissolved

tertiary wall and the secondary walls stains for cellulose (data not shown).

Discussion

As far as we know development of the phytomelan has never been studied by histochemical techniques. Drawings with little detail of seed coat development have been published by Robbins and Borthwick (1925) and Wunderlich (1937). Wunderlich (1937) stated that for *Muscari commosum* and *M. racemosum* “the lumina of the epidermal cells of the outer integument becomes almost completely filled with a dark brown substance”. In *Gasteria* the phytomelan layer consists of outer integumental cells whose lumina are partly replaced by a tertiary wall of phytomelan.

Stage 1: deposition of the tertiary wall

From 14 DAP until 16 DAP deposition of a callosic tertiary wall occurs. In the outer epidermis of the outer integument two thirds of the radial secondary walls thickens, starting at the outer tangential side. The thickening walls stain for cellulose but not for pectin. However, it cannot be concluded that pectin is absent from the radial walls; other wall components might cover the pectin, or there may be too little pectin present to visualize the staining. When the secondary radial wall thickening is complete, pectin staining becomes visible, starting on the outer tangential side of the walls; the radial wall stains for pectin but no longer for cellulose. The treatment of 22 DAP seed sections with hydrogen peroxide-glacial acetic acid

showed that cellulose is still present in the radial walls. Increased pectin or other wall components seem to prevent cellulose staining in these walls. The radial secondary walls do not seem to change any more during subsequent tertiary wall development.

A tertiary wall is deposited against the outer tangential secondary wall, between the thickened parts of the radial secondary walls. The tertiary wall seems to consist of callose and is probably synthesized by plasma membrane-bound callose synthase (Delmer et al. 1993). The callosic tertiary wall stains with PAS, but not with any of the other stains. PAS staining might be the result of the presence of other carbohydrates besides callose, since PAS does not stain β -1,3-glucans (such as callose) (Gabe 1976). The tertiary wall finally occludes about two thirds of the original cell lumen.

Stage 2: first conversion of the tertiary wall

Between 17 and 19 DAP the first conversion of the callosic tertiary wall takes place, resulting in an outer layer bordering the secondary tangential wall, and an inner layer against the plasma membrane. Just before conversion of the callosic tertiary wall can be detected, proteins in the inner layer of the callose become stainable. This inner layer converts into a layer which stains for cellulose and slightly for pectin. The border between the inner tertiary layer and the outer layer is not very distinct and cellulose isotropy suggests that cellulose near the plasma membrane is crystalline. The proteins in the inner layer are only stainable dur-

ing the conversion and therefore may be involved in the formation of cellulose and pectin. Although it is known that cellulose synthase is localized at the plasma membrane (Delmer 1987, Emons 1991), it seems that cellulose and pectin are formed inside the tertiary callosic wall. The callose is probably the carbohydrate source for cellulose formation. Meier et al. (1981) showed that callose might function as an intermediate in the cellulose biosynthesis in cotton fibres. The callose in the outer part of the tertiary wall undergoes a remarkable pattern of conversion. A substrate seems to penetrate the callosic wall via the plasma membrane and radial walls, and interferes with the callose. The consistency of the callose apparently permits this penetration and reaction. The penetrating substance might come from the cytoplasm. The outer layer of the callosic tertiary wall seems to be used during the first conversion as a source of carbohydrates, since there is a distinct border between the newly-formed PAS negative material and callose during the conversion. The callosic wall also seems to serve as a mould for the converted tertiary wall, because the thickness of the callosic wall determines the thickness of the tertiary wall after conversion. Such functions for callose are known from microspore development; the breakdown products of the callosic wall around microspore tetrads are a source of carbohydrates for further microspore development (Keijzer and Willemse 1988) and the callosic tetrad wall can function as a mould for the pollen wall (Waterkeyn and Bienfait 1970, Willemse 1971). However, in this study the callosic wall seems to function simultaneously as a mould and source for the new tertiary wall. The composition of the converted outer layer remains unknown because it does not stain for cellulose, pectin, lignin, phenolics, carbohydrates, or fatty substances. It is also possible that there is nothing to be stained, if during conversion the callosic wall is broken down by enzymes into mono- and disaccharides. Such saccharides cannot be fixed by glutaraldehyde and will be rinsed away during the embedding procedure.

Stage 3: second conversion of the tertiary wall

Starting at 20 DAP a second conversion occurs in the outer layer of the tertiary wall. The whole layer slowly becomes brown and later black (the phytomelan). This darkening process, and the degeneration of the cytoplasm, suggest that the elements required for this conversion are already present in the tertiary layer.

The composition of phytomelan remains unclear, because only toluidine blue stained the outer tertiary layer. The green stain obtained with toluidine blue indicates the presence of phenolics (Feder and Wolf 1965). This suggests that phytomelan is a type of melanin, because phenolics are involved in melanin synthesis (Thomson 1976). The treatment of sections with hydrogen peroxide-glacial acetic acid followed by cellulose staining, produced no reaction indicating the presence of cellulose.

The phytomelan layer around the seed appears to be discontinuous: black tertiary wall material (phytomelan) is separated by radial secondary walls. Phytomelan is chemically very inert and impermeable to water (Werker and Fahn 1975). But breaks in the phytomelan layer at the micropylar opening and hilum, plus its discontinuous nature, raises questions about its function in protecting the seed against fungal and microbial invasion. It is possible that it protects against rapid dehydration and radiation while the micropylar opening and the hilum might be important for water uptake prior to germination. The construction of the phytomelan layer, i.e., stiff phytomelan within a softer secondary wall network, gives this mechanical layer (Takhtajan 1985) more flexibility.

Further studies are in progress to obtain more information on the ultrastructure of the phytomelan layer, and the synthesis and macromolecular composition of the phytomelan.

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