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ScienceDirect
Flora 202 (2007) 261–267
FLORA
www.elsevier.de/flora

The pattern of cuticular blue-fluorescing phenolics deposition during the development of *Prunus persica* leaves

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Received 2 March 2006; accepted 18 August 2006

Abstract

Although stomatal ontogeny is closely related to the development and maturation of the epidermal tissue, stomatal patterns in relation to cuticle construction and cuticular material deposition during leaf development have not received adequate attention. We observed the deposition of blue-fluorescing cuticular phenolics over guard and epidermal cells, as well as stomatal formation and patterning using the alkali-induced blue fluorescence of the cuticle of *Prunus persica* leaves. Stomata of different stages of maturity occurred together during leaf development, mainly at the tip of the lamina. The deposition of fluorescing compounds initially appeared over the guard cells of the developing stomata complexes and gradually extended to the neighbouring epidermal cells. Based on the blue fluorescence emitted by the cuticular layers, we constructed digital maps of leaves of different developmental stages, showing the pattern of stomatal formation and deposition of fluorescing compounds. A longitudinal tip-to-base gradient in the formation of stomata, as well as in the deposition of fluorescing compounds was observed in young developing leaves. The deposition of blue-fluorescing phenolic compounds seems to be coordinated with stomatal development.

Keywords: Cuticle; Leaf development; Phenolics; *Prunus persica* L.; Stomata; Blue fluorescence

Introduction

The surface of aerial plant organs is covered with a series of layers of hydrophobic materials (termed the cuticle) that primarily functions as a waterproof barrier as well as a protection mechanism against environmental stresses (Fahn, 1990; Gniwotta et al., 2005; Kolattukudy, 1996). The ubiquitous presence of the cuticular layers bears testimony to their essential function. Genetic and environmental factors influence wax quantity and composition in the particular organs and tissues, suggesting that the formation of the various cuticular layers is an actively regulated process (Holroyd et al., 2002; Post-Beittenmiller, 1996; Riederer and Markstädter, 1996).

Leaves develop basipetally, with the meristematically active cells occupying a progressively more basal position as the developing cells contribute to the formation of the tip (Dale, 1988; van Volkenburgh et al., 1998). Although monocotyledonous and dicotyledonous leaves are morphologically dissimilar, they develop basically in the same way. In both, the tip matures before the base of the lamina since cells at the

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doi:10.1016/j.flora.2006.08.001

base retain meristematic activity for some time. However, while monocotyledon leaves exhibit polarized growth, leaves of dicotyledons have scattered clonal growth with new cells being intercalated between existing cells (Croxdale, 2000).

Karabourniotis et al. (2001) found that the guard cells of many species, either monocots or dicots, can be identified by an alkali-induced emission of blue fluorescence upon excitation by UV light. The blue fluorescence is mainly derived from the cuticular layer covering both epidermal cells and stomata. Guard cells emit stronger fluorescence than the neighbouring epidermal cells, suggesting that either the guard cell cuticle contains an increased concentration of fluorescing compounds or that guard cells have a thicker cuticle. The lipid layer of the leaf surfaces of *Olea europaea* and *Prunus persica* contain ferulic acid and p-coumaric acid, as well as a number of unidentified compounds esterified to the cuticular waxes that are responsible for the emitted fluorescence (Liakopoulos et al., 2001).

Although the adaptive responses of stomata have long been of interest to plant physiologists, stomatal patterns and their relationship to cuticle construction and cuticular material deposition during leaf development have not received adequate attention. The alkali-induced blue fluorescence of the guard cells offers a rapid and convenient method for *in situ* observation of cuticular characteristics mainly related to stomatal construction. It also permits the visualization of the cuticular matrix. We applied this method in order to examine the deposition of cuticular phenolics over guard cells and epidermis throughout leaf development of *Prunus persica*. Using the above approach we were able to study stomatal formation and patterning during leaf development in relation to cuticle construction.

Materials and methods

Plant material

South-facing developing shoots of *Prunus persica* L. from sun-exposed sites (receiving 1800–2000 μmol photons $m^{-2} s^{-1}$ PAR at midday) at the experimental plantation of the Agricultural University of Athens, Greece, were used as the plant material. Leaf developmental stage was specified by the number of days after emergence (DAE). Samplings were conducted once by collecting leaves of all ages that developed in a single flush under the same environmental conditions, during spring 2004. DAE and average size of the leaves at different developmental stages are given in Table 1. Samples were immediately wrapped in plastic bags, transferred to the laboratory and stored at 4 °C until required for the experiments.

Microscopic observations and construction of stomatal density maps

Observations were conducted with a Zeiss Axiolab fluorescent microscope (Carl Zeiss, Jena, Germany) equipped with a colour CCD video camera (SSC-DC 38p/45, SONY Corporation, Tokyo, Japan). Intact leaf surfaces or leaf sections were treated with KOH solution as described in Karabourniotis et al. (2001). Images were digitally stored in a personal computer. For the construction of stomatal density maps at various developmental stages, leaf surface was divided into equal parts that corresponded to the optical field of the objective lens. The image of each part was recorded and stomata were counted according to Karabourniotis et al. (2001). Since the discrimination between common epidermal cells and stomata at very early developmental stages was difficult, the criterion for a stoma to be included in the count was the delimitation of the stomatal pore by the guard cells. All counts referring to a leaf were combined into a stomatal density map. Mean and maximum stomatal density was calculated on a whole leaf basis (Table 1). All maps (replicates) referring to a specific developmental stage were resized to fit exactly to each other based on the average size of each developmental stage and the mean stomatal density map was calculated.

Measurement of fluorescence intensity of the crude rinses

Fluorescence intensity from the corresponding chloroform rinses of the abaxial surface of *Prunus persica* leaves at various developmental stages was recorded in a FP-920 fluorescence detector (Jasco Corporation, Tokyo, Japan) from 376 to 600 nm (excitation light at 366 nm).

Results and discussion

The deposition of cuticular phenolics during leaf development of *Prunus persica*

Guard cells of mature leaves of *Prunus persica* emit a blue fluorescence upon alkali treatment, brighter than that of the surrounding epidermal cells (indicating the presence of cuticular phenolics, see Karabourniotis et al., 2001; Liakopoulos et al., 2001, see also Figs. 1(A) and 2(E)). Young leaves of *Prunus persica* did not bear dense trichome layers that could prevent the observation of the leaf surfaces by the fluorescence microscope, or emit strong fluorescence under the irradiation field. Microscopic examination of the leaf surfaces showed no glands or glandular hairs, whose