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ORIGINAL PAPER

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Localisation and expression of arabinogalactan-proteins in the ovaries of *Nicotiana alata* Link and Otto

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Abstract The transmitting-tissue cells of the style of flowering plants secrete a complex extracellular matrix through which pollen tubes grow to the ovary to effect fertilisation. This matrix is particularly rich in a class of proteoglycans, the arabinogalactan-proteins (AGPs). AGPs from the ovary of *Nicotiana alata* were found to be developmentally regulated, as the different charge classes of AGPs altered during floral development. The AGPs from the mature ovary had charge characteristics that were distinct from those previously reported for the stigma and style. However, the concentration of AGP (0.6 μg/ml fresh weight) in the ovary did not change during development, or in response to either compatible or incompatible pollination. The AGPs of the ovary are mainly associated with the epidermis of the placenta.

Key words Arabinogalactan-proteins · Ovary · *Nicotiana* · Pollination · Flower development

Introduction

In flowering plants the pistil provides an environment suitable for the germination of pollen and for the growth of pollen tubes through the transmitting tissue to the ovules where fertilisation occurs (Knox 1984). The pathway of pollen tube growth is through an extracellular mucilage which provides a complex growth medium for the pollen tubes. In addition to low-molecular-weight metabolites such as sucrose and amino acids, the mucilage contains a range of glycoproteins including the self-incompatibility ribonucleases (S-RNases; Anderson et al. 1986) and several proline-rich proteins (Chen et al. 1992), a 120-kDa glycoprotein (Lind et al. 1994) and arabinogalactan-proteins (AGPs; Hoggart and Clarke 1984). AGPs have been detected at high concentrations in pistil extracts of many species of flowering plants (Hoggart and Clarke 1984). They may be involved in the adhesion of pollen to the stigma surface, may provide a nutrient supply to growing pollen tubes, may play a role in control of water balance, or could act as antimicrobial or anti-fungal agents (Labarca and Loewus 1972, 1973; Fincher et al. 1983).

AGPs of stigmas and styles of *Nicotiana alata* are developmentally regulated. The amount of AGP in these organs increases during floral development from green bud to mature flower, as shown both by direct quantitation of buffer-soluble AGPs using the β-glucosyl Variv reagent (Gell et al. 1986) and by quantitative immunocytochemistry using a monoclonal antibody directed to terminal α-L-arabinofuranosyl residues (Sedgley and Clarke 1986). Following pollination with either compatible or incompatible pollen, the amount of AGP increases in the stigma, but not in the style, of *N. alata* (Gell et al. 1986). However, neither of these studies investigated changes in expression of AGPs in the ovary. This paper describes the changes that occur in the AGPs in the ovary of *N. alata* during flower development and following pollination.

Materials and methods

Plant material

Seeds of *Nicotiana alata* Link and Otto plants (self-incompatibility genotypes S_2S_2 and S_3S_3) from Dr K.K. Pandey (DSIR, Palmerston North, New Zealand) were grown in pots in a glasshouse as previously described (Gell et al. 1986). *Lycopersicon peruvianum* (L.) Mill. plants were also grown in pots in a glasshouse, and pollen was collected from mature flowers.

All *N. alata* flowers were emasculated at the beginning of petal colouration. Flowers were collected at seven different stages of development: green bud, elongated green bud, petal formation, petal coloration, maturity (indicated by the presence of a sticky exudate on the stigma surface), 24 h post-maturity and 48 h post-maturity. In addition, some flowers were hand-pollinated at maturity and collected 48 h later. Mature stigmas (genotype S_3S_3) were pollinated using either compatible pollen from plants of genotype S_2S_2 or

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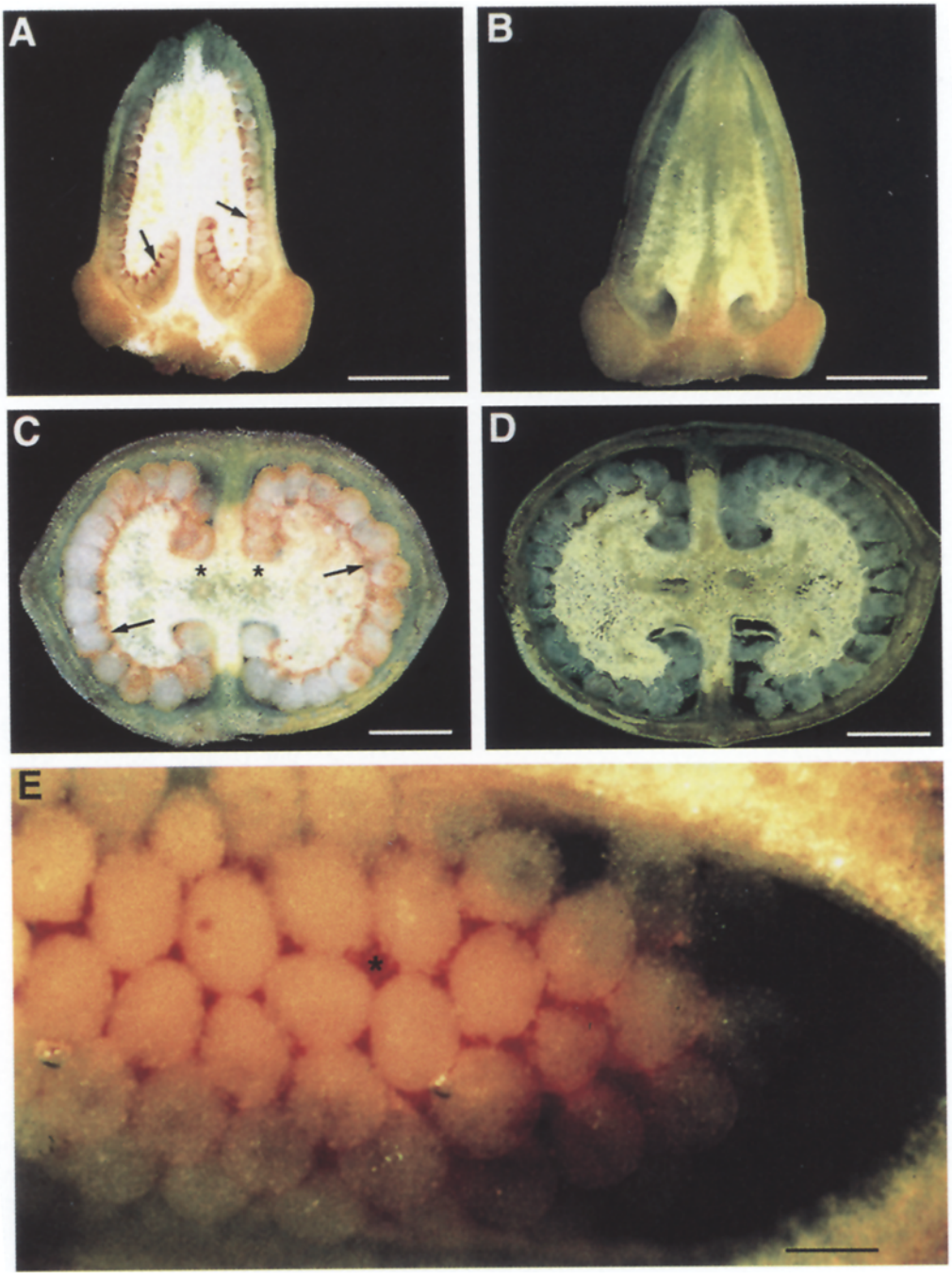

Fig. 1A–E Sections of the mature ovary of *Nicotiana alata* stained with Yariv reagents. **A** Longitudinal section stained with β-glucosyl Yariv reagent, showing staining of the epidermis of the placenta (*arrows*). *Bar* 1.0 mm. **B** Control section stained with α-galactosyl Yariv reagent. *Bar* 1.0 mm. **C** Transverse section stained with β-glucosyl Yariv reagent, showing staining mostly associated with the epidermis of the placenta (*arrows*) and some staining in the vascular tissues (*asterisks*). *Bar* 0.5 mm. **D** Control section stained with α-galactosyl Yariv reagent. *Bar* 0.5 mm. **E** Longitudinal section at higher magnification stained with β-glucosyl Yariv reagent, showing staining associated with the epidermis of the placenta (*asterisk*), but no staining of the ovules. *Bar* 0.2 mm

incompatible pollen from plants of genotype S_3S_3. In control experiments, pistils were either not pollinated; or were pollinated with non-viable pollen (compatible and incompatible) or with synthetic polyacrylamide beads (Biogel P2). Non-viable pollen was prepared by treating pollen with ethanol vapour for 3 h. In each treatment eight flowers were pollinated. One of the flowers was examined microscopically for pollen-tube growth by squashing the pistil and staining with decolorised aniline blue (see below). The remaining seven pistils were dissected from the flowers and AGPs were extracted as described below.

Chemicals

The β-glucosyl Yariv and α-galactosyl Yariv reagents were from Biosupplies Australia (Parkville, Victoria, Australia). Gum arabic and bromophenol blue were purchased from Sigma. Agarose was from Calbiochem. Aniline blue was from Merck. All other chemicals were analytical reagent grade unless otherwise specified.

Histological procedures

Longitudinal sections from ovaries of fresh *N. alata* flowers were cut by hand with a razor blade and used directly in cytochemical tests. Sections were stained for 20 min using β-glucosyl Yariv reagent (2 $mgml^{-1}$ in 1% w/v NaCl) as a bright-field colour reagent, and then washed twice in 1% w/v NaCl. As a control for the staining specificity, sections were treated in the same way with α-galactosyl Yariv reagent, which does not react with AGPs (Jermyn 1978). Sections were viewed with a Wild Photomakroscop M400 and an Intralux 150H lighting unit. The extent of pollen-tube

growth in pollinated pistils was measured after staining the pollen tubes with decolorised aniline blue as described by Webb and Williams (1988a).

Preparation of tissue extracts from pistils

Seven pistils were collected from flowers at different stages of development or from pollinated flowers, and the ovaries were removed with a razor blade. The seven ovaries were weighed, then frozen in liquid nitrogen and ground in an Eppendorf tube to a fine powder. Buffer-soluble components were extracted with 200 µl of 0.2 M TRIS-HCl (pH 8.0) containing 10 mM NaCl, 10 mM EDTA, 1 mM DTT, and 0.5% v/v Nonidet P-40 (Sigma). The homogenate was centrifuged at 10000 *g*, 4° C for 10 min. An aliquot of the supernatant was then assayed for AGPs using the radial diffusion assay (van Holst and Clarke 1985). The remainder of the supernatant was dialysed against distilled water using micro-colloidan bags (M_r cut-off 12000; Sartorius) and freeze dried.

Radial diffusion assay for the quantification of AGP in tissue extracts

The amount of AGP in tissue extracts was measured using the radial diffusion assay against β-glucosyl Yariv reagent (van Holst and Clarke 1985) with gum arabic (0.25–3.0 µg; Sigma) used as a standard. This method incorporates β-glucosyl Yariv reagent into an agarose gel. The reagent interacts with and precipitates AGP as it diffuses through the gel. Diameters of the precipitation rings formed around the wells were measured to within 0.1 mm with a calibrated eyepiece. There was a linear relationship between the amount of AGP in a sample and the area of precipitation, which was measured as the square of the diameter of the halo. The amount of AGP in test samples was calculated with reference to a standard curve for gum arabic (µg) versus the square of the measured diameter in millimetres. Duplicates were reproducible to within ±2%.

Crossed electrophoresis

The different charge families of AGP present in tissue extracts were demonstrated by crossed electrophoresis performed according to van Holst and Clarke (1986). The movement of AGPs was measured relative to the bromophenol blue dye front (R_F), and peak mobilities were reproducible to within ±4%.

Statistical treatment of data

A minimum of three separate tissue extracts were made for each data point, and the arithmetic mean and standard deviation were calculated. The distributions of the data collected for the amounts and contentrations of AGP in the tissue extracts were statistically normal (i.e. 95% of the values were within two standard deviations of the mean). Analysis of variance (ANOVA) was used to test the data for significant differences between the means, with a null hypothesis that the samples could have been drawn from a single population. The Student-Newman-Keuls procedure (Sokal and Rohlf 1969) was then used to assess which mean values were significantly different.

Results

Localisation of AGPs in ovaries of *N. alata*

The ovary of *N. alata* has two loculi separated by a septum, and the ovules are attached to a central placenta (Figs. 1A–D). The transmitting tissue enters the top of the ovary and divides into two strands each reaching one of the two locules and becoming continuous with the epidermis of the placenta. Sections of mature ovaries stained with the β-glucosyl Yariv reagent showed that staining was mainly associated with the epidermis of the placenta (Figs. 1A, C); staining was not observed within the ovules (Fig. 1E). There was also some staining of the vascular tissue (Fig. 1C). No staining was observed in the control sections with α-galactosyl Yariv reagent (Figs. 1B, D). Ovaries stained with β-glucosyl Yariv reagent 48 h after pollination treatments showed the same pattern of staining (not shown).

Table 1 Amounts and concentrations of AGPs in the ovary of *Nicotiana alata* at different stages of floral development. The values represent the mean of at least three separate tissue extracts. *FW* Fresh weight. Different superscript letters in the same column denote results that are significantly different ($P<0.05$)

Stage of development	AGP	
	Amount (µg)	Concentration (µg/mg FW)
Green bud	3.4^{a}	0.8^{c}
Elongated green bud	$4.7^{a,b}$	0.9^{c}
Petal formation	$5.3^{a,b}$	0.7^{c}
Petal coloration	5.9^{b}	0.6^{c}
Maturity	5.5^{b}	0.5^{c}
Post-maturity (24 h)	7.1^{b}	0.6^{c}
Post-maturity (48 h)	6.8^{b}	0.6^{c}

AGPs in developing ovaries of *N. alata*

The amounts and concentrations of AGP in buffer-soluble extracts of ovaries from unpollinated pistils at different stages of floral development are shown in Table 1. The amont of AGP in the ovary increased during maturation of the ovary, and up to 24 h post-maturity (Table 1). However, the increase in the amount of AGP was proportional to the increase in the fresh weight (FW) of the ovary, and the concentration of AGP in the ovary thus remained approximately constant at 0.6 µg AGP mg^{-1} FW.

The different charge classes of AGP present in the ovary throughout floral development were examined by crossed electrophoresis (Fig. 2). At the green bud stage the pattern of AGPs was diffuse with respect to charge and no peaks were resolved. After the green bud stage, the crossed electrophoresis pattern of AGPs from the ovary began to resolve; at the elongated green bud stage two diffuse groups of AGPS were detected (R_F 0.26 and 0.50). At maturity and post-maturity the charge properties of the AGPs from the ovary again changed; one major peak (R_F 0.41) and one minor peak (R_F 0.84) could be resolved.

AGPs in ovaries of pollinated pistils of *N. alata*

The amounts of AGP in the ovary 48 h after various pollination treatments are shown in Table 2. There was a

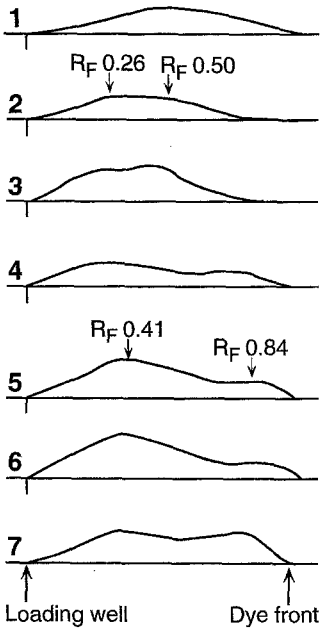

Fig. 2 Crossed electrophoresis of AGPs in the ovary of *Nicotiana alata* during floral development and following a compatible pollination. Ovary extracts were from the following developmental stages: *1* green bud, *2* elongating green bud, *3* petal formation, *4* petal coloration, *5* maturity, *6* 48 h postmaturity, *7* following compatible pollination

significant increase in the amount of AGP only when flowers were pollinated with compatible pollen. However, after a compatible pollination and subsequent fertilisation the ovary swells, and the increase in the amount of AGP was proportional to the increase in the fresh weight of the ovary tissue. Thus, the concentration of AGP in the ovary did not change significantly with any of the pollination treatments.

The crossed electrophoresis patterns of AGPs present in the ovary were not changed by any of the pollination treatments as compared to unpollinated controls, except that in compatible pollinations the relative proportions of the two classes of AGP are altered; relatively more AGPs of R_F ~0.84 are present (Fig. 2).

Discussion

During fertilisation in flowering plants with closed styles, such as *Nicotiana alata*, pollen tubes grow extracellularly down between the stigmatic papillae, through the transmitting tissue in the style and over the epidermis of the placenta in the ovary to reach the ovulus. When pollen grains land on a mature stigma, the mucilage in which they land contains AGPs, as does the extracellular mucilage of the transmitting tissue in the style. AGPs have also been detected in the extracellular mucilage of the stigma and style of *Gladiolus gandavensis, Lilium longiflorum, Lycopersicon peruvianum* and *N. alata* (Clarke et al. 1978; Gleeson and Clarke 1979; 1980; Sedgley et al. 1985; Sedgley and Clarke 1986; Webb and Williams 1988b). We show here that AGPs are also present at the epidermis of the placenta. Thus, the tissues through which pollen tubes grow all produce AGPs. These tissues are derived from the inner epidermal layer of the developing carpels.

During the development of *N. alata* flowers from green bud to maturity the amount of AGP in the ovary increases but the concentration remains almost constant (Table 1). This observation is similar to that reported for AGPs in the style of *N. alata*, but contrasts increased concentration of AGP in the stigma at maturity (Gell et al. 1986). This increase in AGPs in the stigma coincides with the appearance of a sticky exudate on the stigma surface, and with the stigma becoming receptive to pollen (Gell et al. 1986).

The crossed electrophoresis experiments indicate that the AGPs in the ovary of *N. alata* change in their charge characteristics during flower development (Fig. 2). At the green bud stage the diffuse electrophoretic pattern of the AGPs in the ovary is similar to that previously reported in the stigma and style (Gell et al. 1986). As the flowers develop, however, the crossed electrophoresis patterns of the AGPs from the three organs change and in mature pistils the stigma, style and ovary each have a distinct charge profile of AGPs (Fig. 2; Gell et al. 1986). Following pollination with either compatible or incompatible pollen, there are no changes in the charge classes of AGPs in the stigma, style or ovary, although there are changes in their relative proportions (Fig. 2; Gll et al. 1986). The different charge patterns seen in the stigma, style and ovary during floral development and after fertilisation probably reflect differences in the charge of the protein core of the AGPs, as we have shown that there are no uronic acids in the carbohydrate moiety of AGPs from *N. alata* stigmas and styles (Gane 1994; Gane et al. 1994). Recently, cDNAs encoding the protein backbones of AGPs from stigmas and styles of *N. alata* (Du et al. 1994) and suspension-cultured cells of *Pyrus communis* (Chen et al. 1994) have been described. These studies show that the protein back-

Table 2 Amounts and concentrations of AGPs in the ovary of *Nicotiana alata* following different pollination treatments. The values represent the mean of at least three separate tissue extracts. FW Fresh weight. Different superscript letters in the same column denote results that are significantly different ($P<0.05$)

[1] Pollen killed with ethanol vapour prior to pollination

	Test cross	AGP	
		Amount (μg)	Concentration (μg/mg FW)
Unpollinated	S_3S_3 ♀	6.8[a]	0.6[c]
Compatible	S_3S_3 ♀ × S_2S_2 ♂	12.1[b]	0.8[c]
Incompatible	S_3S_3 ♀ × S_3S_3 ♂	7.9[a]	0.6[c]
Control compatible	S_3S_3 ♀ × S_2S_2 ♂ (non-viable)[1]	7.2[a]	0.5[c]
Control incompatible	S_3S_3 ♀ × S_3S_3 ♂ (non-viable)[1]	8.8[a]	0.7[c]
Interspecific	S_3S_3 ♀ × *L. peruvianum* ♂	8.1[a]	1.0[c]
False pollination	S_3S_3 ♀ × synthetic beads ♂	7.4[a]	0.7[c]

bones of AGPs from a particular tissue can have different isoelectric points, which could account for the range of electrophoretic properties described in this study.

The function of AGPs in flower development and fertilisation is still unclear. During the interval from petal coloration to maturity the pistil becomes receptive to pollen and an exudate rich in AGPs is produced on the stigma surface, which is thought to aid the trapping of pollen and prevent the stigma from becoming desiccated. The extracellular matrix within the pistil tissues must provide a suitable medium for pollen-tube growth. It has been suggested that AGPs may be a source of nutrition for the growing pollen tubes (Labarca and Loewus 1972, 1973). The extracellular mucilage may also provide physical support for the pollen tubes as well as a pathway of minimum resistance for the extension of the delicate pollen-tube tip, since the AGPs are high-molecular-weight proteoglycans with a high water-holding capacity that contribute to the physical characteristics of the viscous matrix. Sanders and Lord (1989, 1992) have suggested that the extracellular mucilage may also play an active role in directing pollen-tube growth, and they propose that vitronectin-like proteins in the mucilage may function in pollen-tube extension (Sanders et al. 1991). A similar or related role for AGPs cannot be excluded, since AGPs have been functionally implicated in cell-cell interactions (Pennell et al. 1991; Knox 1992; Pennell 1992). The recent cloning of a gene encoding the protein backbone of an AGP from *N. alata* pistils (Du et al. 1994) will allow us to address these questions.

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