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Pollen viability and transgene expression following storage in honey

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Transgenic plants of tobacco and *Arabidopsis* that produce genetically marked pollen, expressing the reporter gene *uid*A (*gus*A), were generated to determine whether pollen proteins can be expressed and stable in honey, a potential route by which foreign proteins might enter the wider environment. Hydrated tobacco pollen was found to lose viability rapidly in honey, while pollen in the natural dehydrated form remained viable for at least several days and in some cases several weeks, as determined by FDA staining activity and germinability. Dehydrated pollen was found to be capable of transient foreign gene expression, following microprojectile bombardment, after incubation in honey for at least 120 h. PCR amplification of transgene sequences in pollen of transgenic plants revealed that pollen DNA can remain relatively intact after 7 weeks in honey. GUS enzyme activity analysis and SDS–PAGE of pollen proteins revealed that foreign and native pollen proteins are stable in pollen incubated in honey for at least 6 weeks. We conclude that pollen may represent an ecologically important vector for transgenic protein products.

Keywords: Transgenic plants; pollen gene expression; β-glucuronidase (GUS) activity; environmental impact of transgenics; molecular ecology

Introduction

The genetic manipulation of arable crops has advanced rapidly since the establishment of vector-mediated and direct gene transfer systems for plant transformation. The feasibility of the release of transgenics into the wider environment, for agricultural exploitation, brings into focus questions of the possible environmental impact of the dispersal of foreign genes and the protein expression products in pollen. While cross-pollination with wild species represents a potential route of genetic dispersal (Dale, 1992; Scheffler *et al.*, 1993; Cresswell, 1994; McPartlan and Dale, 1994), the protein gene products, some of which might have physiological activity in animals, have to date received less consideration (but see Nap *et al.*, 1992). A wide diversity of genes is now being expressed in transgenic plants, including genes encoding insecticidal proteins (Gatehouse *et al.*, 1992; Williams *et al.*, 1992), enzymes and other commercially interesting proteins (Sijmons *et al.*, 1990; Oakes *et al.*, 1991), cereal seed storage proteins (Hoffman *et al.*, 1987), animal hormones (Vandekerckhove *et al.*, 1989) and antibodies (Hiatt *et al.*, 1989; Owen *et al.*, 1992). Potentially, these proteins may incur physiological effects on insect populations (notably the *Bacillus thuringiensis* toxin or cowpea trypsin inhibitor); or, for some proteins (e.g. allergenic cereal storage proteins such as gluten), humans and other animals, following either entry into the food chain as components of bee-transmitted pollen in honey, or inhalation with airborne pollen grains (Fox, 1994).

In order to investigate the possible significance of pollen as a route by which biologically functional foreign proteins may enter the environment, our strategy was to generate transgenic plants that express a reporter transgene, *uid*A (*gus*A) in pollen. We have previously generated transgenic tobacco containing this reporter gene under the transcriptional control of the pollen-specific tomato gene promoter *lat59* (Twell *et al.*, 1990); and *Arabidopsis* plants containing a promoterless *gus*A gene activated by random insertion downstream of a native pollen gene promoter (Lindsey *et al.*, 1993). Both sets of plants therefore produce genetically marked pollen that can be studied under a range of environmental conditions. The aim of the study was to investig-

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ate the viability of transgenic pollen incubated in honey, and to determine whether a pollen-expressed transgene product (GUS enzyme) can remain stable in honey.

Materials and methods

Plant material

In order to generate transgenic plants that produce genetically-marked pollen, a chimaeric gene, *lat59-gus* (Twell *et al.*, 1990), was introduced into tobacco plants essentially as described by Horsch *et al.* (1985). The gene comprised the *uid*A (*gus*A) coding region, encoding the β-glucuronidase (GUS) protein (Jefferson *et al.*, 1987), under the transcriptional control of the tomato *lat59* gene promoter, which has been shown to be preferentially active at high levels in pollen of several species. This in turn was linked to a selectable *nptII* gene which confers kanamycin resistance to transgenic plants (Twell *et al.*, 1990). T_2 plants homozygous for *lat59-gus* at a single genetic locus (as determined by a 3:1 ratio of kanamycin resistant : sensitive seedling progeny) were grown under greenhouse conditions to maturity, and showed a high level of GUS activity in 100% of the pollen grains (as expected for plants homozygous for the *gus*A gene). Pollen from these plants and from non-transformed tobacco was harvested and stored at −80 °C for use in subsequent experiments.

Non-transgenic pollen of *Brassica napus* was isolated from the flowers of locally grown field-grown plants. Dehiscing anthers were placed in Eppendorf tubes and shaken on a vortex mixer to release the pollen. Anther tissue was removed and the pollen was stored at −80 °C for use in subsequent experiments. Pollen was also isolated directly from local hives, sited adjacent to fields of *B. napus*, after incubation in pollen cells for 2–3 months.

Determination of pollen viability

The effect of incubation in honey on the viability of pollen was assessed by two techniques: by quantifying the germination of pollen *in vitro* and by the use of the vital stain fluorescein diacetate (FDA; Heslop-Harrison and Heslop-Harrison, 1970). *In vitro* pollen grains were grown in pollen germination medium (PGM) at a density of 1×10^4 grains per ml at room temperature. The percentage of pollen grains forming tubes was scored after 4–6 h by microscopic observation. Pollen was used either in its naturally dehydrated state or when hydrated by a 5 min pretreatment in PGM ($CaCl_2$ 300 mg l^{-1}; H_3BO_3 100 mg l^{-1}; 10% w/v sucrose; pH 6.4). For fluorescein diacetate (FDA) staining activity, pollen was first mixed with commercial honey (Spar clear honey, produce of more than one country) at a density of approximately 10^6 grains per ml, and incubated at room temperature. Prior to staining, pollen was reisolated by dissolving the honey in PGM, followed by centrifugation for 30 s at 6500 rpm and removal of the supernatant. The pollen pellet was then resuspended in the PGM containing FDA (0.5 mg ml^{-1}) for analysis. After staining for 5 min, strongly fluorescent grains (excitation wavelength of 490 nm) were scored as viable.

GUS enzyme activity determination

Pollen was incubated in honey at a density of 10^6 grains per ml; reisolated as described; and protein was extracted in GUS extraction buffer (Jefferson *et al.*, 1987). Incubation of pollen in honey at this density is within the range of pollen densities found naturally in honey (Maurizio, 1975). GUS enzyme activity in crude protein extracts of hydrated and dehydrated pollen was determined using 4-methylumbelliferyl glucuronide (Sigma) as substrate, and the fluorescent product 4-methylumbelliferone (4-MU) was assayed using a Perkin Elmer LS-50 luminescence spectrometer (excitation 365 nm, emission 455 nm). Protein concentration was determined using the Bradford reagent (Biorad) according to the manufacturer's instructions. GUS enzyme activity was expressed as nmol 4-MU produced per min per pollen grain.

Transcriptional and translational activity of pollen

Net transcriptional and translational activity of pollen was determined by transient gene activity studies using microprojectile bombardment, following incubation in honey at a density of 20 mg ml^{-1}. Pollen was reisolated from honey and resuspended in 1 ml PGM. 0.5 ml of the suspension was bombarded with the plasmids plat52-7 (encoding the GUS enzyme, under the transcriptional control of the tomato pollen gene promoter *lat52*; Twell *et al.*, 1990) and pNBL-52-5, encoding firefly luciferase and also driven by the *lat52* gene promoter (unpublished) as described previously (Twell *et al.*, 1990). Hydrated and dehydrated non-transgenic pollen was incubated in honey for up to 150 h, reisolated and then bombarded. The combined transcriptional and translational activities of treated pollen were assayed as increases in GUS or LUC activities respectively. Bombarded pollen was incubated for 18 h at 28 °C and resuspended in 1.5 ml extraction buffer (0.1 M KH_2PO_4, 0.1 mM dithiothreitol). GUS and luciferase enzyme assays were performed on crude protein extracts, essentially as described previously (Ow *et al.*, 1986; Jefferson *et al.*, 1987).

PCR amplification of DNA in pollen

Pollen was isolated from *Arabidopsis thaliana* plants that were either non-transformed or were homozygous for a single copy *gus*A gene, following activation of a promoter trap (line AtPS-2; Lindsey *et al.*, 1993). Pollen was

incubated in honey and the stability of the foreign DNA was determined by PCR amplification of the transgene sequence. To isolate DNA for PCR, approximately 10^5 pollen grains were reisolated from honey, frozen on dry ice and ground in 50 µl extraction buffer (200 mM tris-HCl, pH 7.5, 200 mM NaCl, 25 mM EDTA, 0.5% w/v SDS), followed by centrifugation at 13 000 rpm for 1 min. To 40 µl supernatant was added an equal volume of isopropanol; the DNA precipitate was pelleted in a microfuge and vacuum dried. The pellet was redissolved in 10 µl of TE and 0.5–2 µl used for PCR amplification. Reaction mix: 2.5 µl × 10 extraction buffer; 1 µl of each primer (50 ng ml^{-1}); 0.2 µl of *Taq* polymerase (Promega); 0.5–2 µl of template; 5 µl of dNTPs, made up to 25 µl with sterile distilled water. PCR cycles: 95 °C for 5 min, during which time dNTPs were added followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. Amplified fragments were detected by electrophoresis on 1% agarose gels.

Sequences of primers used: (a) −48 primer: 3′AGCGGATAACAATTTCACACAGGA5′; (b) N3 primer: 5′GGACTGGCATGAACTTCG3′.

Protein gels

Protein content of non-transformed dehydrated pollen was determined qualitatively after incubation in honey for (a) 0 weeks, (b) 2 weeks and (c) 6 weeks. Total soluble protein of reisolated pollen was extracted in buffer (100 mM Tris-HCl; 5 mM dithiothreitol; 5 mM EDTA; 0.02% v/v phenylmethylsulphonyl-fluoride; pH 8.0), separated in SDS-denatured protein gels, and stained with Coomassie blue according to Laemmli (1970).

Results

Pollen viability in honey

The data in Fig. 1A describe the viability in honey of pollen, supplied in both the naturally dehydrated condition (as it is found at anthesis and following transport by bees to the hive), and also in the hydrated form (pollen hydration occurs gradually during incubation in honey, over a period of 3–7 days). Whilst dehydrated pollen only gradually lost its viability over several days (approx. 20% of grains viable at 96 h), hydrated pollen exhibited viability of only 0.3% of the grains after 3 hours in honey. Garden flower honey, which was obtained commercially and had been harvested 2 months previously from a local source, contained approximately 10^5 pollen grains per ml. FDA analysis of this pollen demonstrated that approximately 1% of the grains were viable, as indicated by fluorescence staining, and some grains (<1%) were capable of germination.

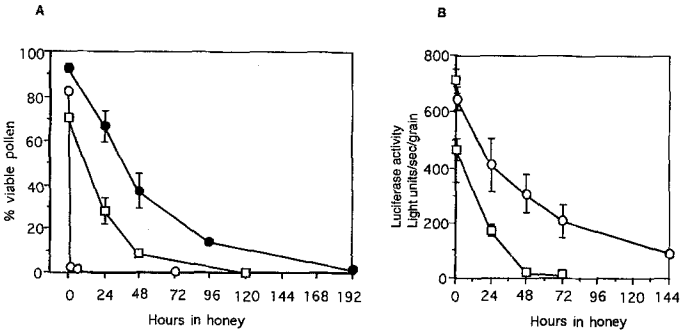

Fig. 1. Viability of hydrated and dehydrated pollen in honey. A. Pollen viability assessed by germinability and FDA staining after incubation in honey. (□) = germinability of dehydrated pollen; (●) = FDA staining of dehydrated pollen; (○) = FDA staining of hydrated pollen. B. The capacity of hydrated and dehydrated pollen, after incubation in honey to transcribe and translate plat52-luc DNA delivered by microprojectile bombardment. (○) = dehydrated pollen bombardment with plat52-luc; (□) = hydrated pollen bombardment with plat52-luc.

Transient gene expression in pollen following incubation in honey

To investigate the effect of incubation in honey on the ability of pollen to transcribe and translate a foreign gene, the transient expression of a pollen-specific promoter–reporter gene fusion was analysed, following the transfection of pollen by microprojectile bombardment (Twell *et al.*, 1989). The transient expression profiles of the dehydrated and hydrated pollen, shown in Fig. 1B, followed closely the viability profiles and show, as expected, that transient expression did not occur in grains designated inviable by a lack of FDA staining. Dehydrated pollen grains were still capable of transient expression after incubation for 120 h in honey whilst hydrated pollen, after incubation for 48 h, contained only a few grains capable of transient expression. The histochemical localization of GUS activity in bombarded pollen grains, using X-Gluc as substrate (Jefferson *et al.*, 1987), showed that the intensity of staining in individual grains expressing the GUS construct was similar, irrespective of time spent in honey (data not shown). It can therefore be concluded that the reduction, with time, in transient GUS expression in pollen incubated in honey was caused by a decrease in the number of grains capable of expressing, and not the expression rate of individual grains.

Stability of pollen DNA following incubation in honey

To investigate the stability of DNA in pollen incubated for up to 7 weeks in honey, the polymerase chain reaction was used to determine whether specific sequences of known lengths could be amplified after

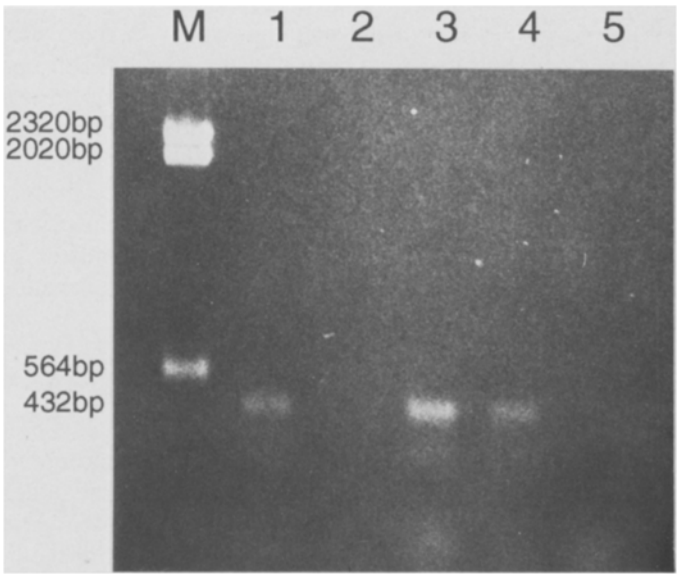

Fig. 2. Integrity of DNA fragments in pollen incubated in honey. Track M = MW markers; track 1 = amplification product from purified genomic DNA of transgenic *A. thaliana* plants homozygous for *gus*A (AtPS-2); track 2 = amplification of purified genomic DNA of non-transformed *A. thaliana* plants; track 3 = amplification product from purified genomic DNA of transgenic AtPS-2 pollen; track 4 = amplification product from purified genomic DNA from transgenic AtPS-2 pollen, incubated in honey for 7 weeks; track 5 = amplification of purified genomic DNA from pollen of non-transformed *A. thaliana* plants, incubated in honey for 7 weeks.

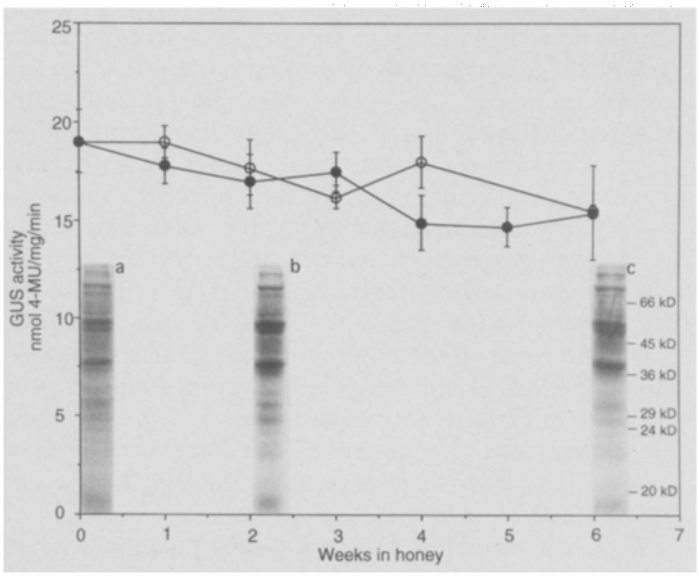

Fig. 3. GUS enzyme activity and protein profile in hydrated pollen (○) and dehydrated pollen (●) extracts following incubation in honey for up to six weeks.

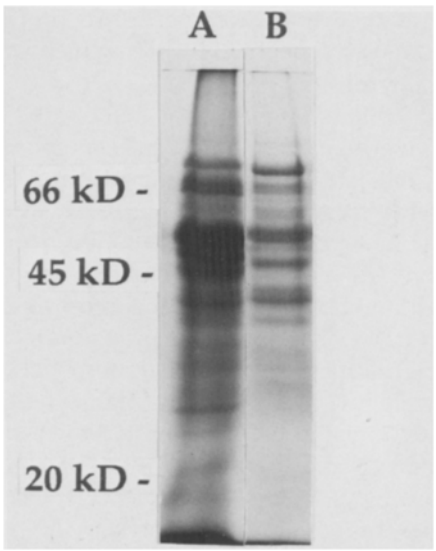

Fig. 4. Protein profiles of pollen isolated from the pollen cells of bee hives adjacent to fields of *B. napus*, two to three months after flowering (A) and from flowers of *B. napus* (B).

incubation periods of increasing duration. The results in Fig. 2 demonstrate that, after 7 weeks in honey, predicted fragments of 432 bp remained intact in the pollen.

Stability of pollen proteins following incubation in honey and in beehives

To investigate pollen protein stability in honey, GUS enzyme activity and qualitative profiles of extractable endogenous proteins in transgenic *lat59-gus* tobacco pollen were determined over a prolonged period of incubation in honey; the results are presented in Fig. 3. Both extractable GUS activity and the endogenous protein profiles, determined following separation on SDS–PAGE gels, remained stable over a 6-week incubation period. No significant difference was observed between the GUS data from hydrated or dehydrated pollen. The maintenance of a high level of GUS activity was unexpected as GUS protein has a half-life of approximately 60 h in tobacco mesophyll protoplasts (Jefferson *et al.*, 1987).

Analysis of the endogenous protein profiles of untransformed *Brassica napus* pollen collected directly from the plant and also from beehives (following 2–3 months' incubation within the hive) is illustrated in Fig. 4. The results demonstrate that, even in pollen that was collected by bees and stored within the hive pollen cells (Fig. 4a), most of the major proteins found in pollen collected directly from the flower (Fig. 4b) remain. These data indicate that contact with bees and incubation in pollen cells within the hive has little significant effect on protein stability in pollen.

Discussion

In this paper we describe the use of an *in vitro* system to investigate the possibility that pollen-expressed proteins

remain functional in honey. This *in vitro* system, in which pollen was mixed directly with honey and incubated for known durations, has two principal advantageous features: first, pollen of a genetically defined constitution (i.e. expressing a characterized reporter gene) can be introduced into honey; and second, the concentration of the pollen can be manipulated, so that detectable levels of reporter enzyme activity and pollen protein profiles can be characterized following treatment with honey. The use of naturally produced pollen/honey mixtures would not satisfy these criteria: transgenic plants expressing high levels of reporter gene activity in pollen are not available in current field experiments, and, in any case, the protein activity in honey derived from such plants is expected to be too low to generate meaningful enzyme activity data.

While the concentration of a given, potentially toxic pollen-borne protein is expected to be very low in natural honey made from nearby transgenic plants, it is nevertheless the case that vanishingly small quantities of allergenic proteins can cause adverse responses in allergic individuals (discussed by Fox, 1994), and our data indicate that at least some pollen proteins may remain intact and/or functional following incubation in honey for several weeks.

The pollen viability data, determined by staining with FDA, germinability and transient gene expression analyses, indicate that pollen survival in our *in vitro* experimental system is low, particularly when incubated in honey in the dehydrated state. We hypothesise that the observed difference in viability between the hydrated and dehydrated pollen (Fig. 1) was most likely caused by the osmotic shock experienced by hydrated pollen upon mixing with honey. Dehydrated pollen, being of a relatively low water content (Stanley, 1971), is presumed not to be subjected to such a shock and consequently retained its functionality for a longer time in honey. Interestingly, we did observe that a small but significant number of pollen grains, present in commercially obtained garden flower honey (2 months post harvest), were capable of germination and stained as viable in FDA.

These results, and the data demonstrating that pollen DNA can remain relatively intact even after 7 weeks in honey, indicates that genes may be expressed in pollen in honey, at least for a few days following incorporation into honey. The protein data, moreover, demonstrate that pollen GUS activity was stable in honey for several weeks, and the major native pollen proteins were similarly present for prolonged periods (Fig. 3). Although our experiments were primarily carried out *in vitro*, and the pollen was not subject to possible degradation by bee proteases, the data presented in Fig. 4 indicate that transport by bees does not significantly affect the protein profile of *Brassica* pollen. Furthermore, Marshall and Williams (1987) have observed that pollen-derived proteins can remain in honey that is commercially sold, and so commercial treatment of the honey (e.g. pasteurization) clearly does not destroy all pollen proteins. It is also the case that not all commercially available honey is heat-treated or otherwise treated to remove protein (Townsend, 1975), and indeed the reduced activity of endogenous honey enzymes is used as an indicator of unacceptable overheating of honey prior to marketing (Fasler, 1975).

Conclusions

In view of the observed stability of transgene and native gene products in pollen, it can be concluded that pollen therefore represents a route by which the protein products of transgenes might be released from transgenic plants to a wider environment. The relatively high concentrations of pollen in our *in vitro* system, and the high activity of the *lat52-gus* reporter gene in tobacco pollen, provides a 'worse case' scenario for the presence of transgene products in pollen, but the data do indicate that at least some pollen proteins are stable in honey. As ever increasing numbers of genetically engineered crop plants are being approved for release experiments, it is vital that the potential problems associated with the expression of transgenic products in pollen are addressed. In particular, it is possible that transgenic crop plants expressing a toxic or allergenic protein in pollen could pose problems, not only to man who consumes honey as a food, but also to bee populations, which rely on pollen as the sole source of protein. We are currently addressing the issue of whether various commonly used regulatory sequences, which can drive the expression of foreign proteins in crop plants, are active at significant levels in pollen, and so whether there are ecological implications of their use. As a strategy to avoid such potential problems, we are also investigating the use of antisense RNA technology to control the ectopic expression of transgenes in pollen.

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