Title of project:	Healthcare products from fruit; delivery of antimicrobial agents in apple to counter tooth decay
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Grower Summary

Background

Streptococcus mutans, which is only found in humans, has a significant role in the development of dental caries. *S. mutans* colonises the surface of teeth using a 'binding' protein (SA I/II) to attach itself. Charles Kelly's group at Guy's Hospital have shown that by applying a synthetic protein (identical to SA I/II) to the surface of the teeth, they can 'block' the attachment of the actual pathogen, by occupying all the sites where the pathogen might bind to the teeth. The 'receptor blocking strategy' is designed to physically prevent the initial infection step of a human pathogen, and may present an alternative therapeutic strategy to antibiotics in the future.

Our project, co-funded by the HDC, and formerly the APRC, and the East Malling Trust for Horticultural Research, aimed to express this protein (SA I/II) in plants, to demonstrate that a protein, which could be used therapeutically, can actually be produced in a transgenic plant. We have put the SA I/II transfer gene into *Agrobacterium*, and have carried out two major transformation experiments to transfer this gene into apple (cv Greensleeves).

Results & potential benefits

The project has been partially successful, as five putative transgenic lines are currently being screened, at least one of which has been shown by PCR to contain the entire SA I/II gene. Further PCR analysis, coupled with Southern Analysis will continue beyond the official completion date of this project, and will be undertaken by Professor David James of EmPharm. Further work will then be necessary to determine whether the inserted gene can successfully produce the protein in the apple fruits.

This research programme has provided an important opportunity to develop a collaborative research programme with Guy's Hospital on the production of plant-derived vaccines for human healthcare. In the longer term, more extensive research programmes in this area may benefit the fruit growing industry through the development of strategies for the genetic modification and production of a range of human therapeutic proteins on a large scale. It is anticipated that this work will act as a demonstration project for future work with horticultural crops.

Science Section

Background

Anti-adhesive strategies for disease prevention at mucosal surfaces

The clinical effectiveness of most antibiotics has declined dramatically in recent years as organisms have become increasingly resistant to them, resulting in a situation where the use of antibiotics is becoming seriously compromised (Neu 1992). One of the alternative therapeutic strategies for combating infectious disease which does not involve the use of antibiotics is the 'receptor blocking strategy', designed to prevent the initial infection step of the pathogen. This involves the adherence of the pathogen, by specific microbial adhesions to the mucosa of the oro-intestinal, nasorespiratory or genitourinary tracts. This step is critical in the process of microbial infection. Whilst adhesion may not be an absolute requirement for infection, it is an essential step in the development of pathology (Kelly and Younson 2000).

There are three main classes of adhesion-blocking antimicrobial agents; receptor analogues, adhesion analogues and anti-hesion antibodies. Anti-adhesion antibodies could prevent adhesion of the pathogen by blocking the access to the host analogue. Receptor and adhesion analogues could act as competitive inhibitors of adhesion. Most work which has been carried out on these approaches has focussed on the prevention of infection at mucosal surfaces (Kelly and Younson 2000). This is due to the role of mucosal surfaces as a main entry point into the body of infectious diseases, and also their accessibility for the delivery of adhesion blocking antimicrobial agents.

An important longer term advantage of using adhesion blocking agents is that microbial resistance is much less likely to develop, in comparison to treatment with antibiotics. This is because simply blocking pathogen binding will not provide the sustained selective pressure which occurs during treatment with antibiotics.

Oral hygiene

The human mouth is colonised by a diverse flora comprising species of fungi, protozoa, viruses and bacteria. Whilst the majority are commensals, pathogenic microorganisms have been identified that give rise to the two major oral diseases, namely dental caries and peridontitis. In particular, *Steptococcus mutans*, which is only found in humans, has been identified as the primary etiological cause of dental caries. Dental caries remains a major public health problem, with the incidence of dental caries as high as 45% in five year old children (1993 Children's Dental Health National Survey). In 1998, the cost of dental fillings to the NHS was approximately £210 million.

S. mutans, is not readily controlled through conventional oral hygiene techniques. Colonisation of the surface of teeth is mediated by a non-fimbrial cell surface adhesin of *S. Mutans* termed the streptococcal antigen I/II (SA I/II). This cell surface adhesin binds to salivary agglutinin and other components of the glycoprotein pellicle layer which is bound to the mineral matrix of the teeth. Inactivation of the SA I/II adhesin has been shown to prevent colonisation and hence dental caries (Ma *et al* 1989). An adhesion epitope has been identified on the SA I/II adhesion, mapped to residues

1025-1044 (Kelly *et al* 1995). More recently, Charles Kelly's group at Guy's Hospital have developed a novel 'receptor block' approach to prevent infection by *S. mutans*, by topical application.

Binding of the SA I/II adhesin to salivary agglutinin has been inhibited *in vitro* by a synthetic peptide (p1025), which corresponds to residues 1025-1044 of the adhesin (Kelly *et al* 1999). Human trials with p1025 have shown that following a small dose, recolonisation was prevented for 88 to >120 days. Larger peptides containing this adhesion epitope, that have adhesion activity (Munro *et al.,* 1993; Kelly *et al.,* 1995; Kelly *et al.,* 1999) have been selected, spanning residues 803-1185 (fragment 1) and residues 820-1538 (fragment 2). This mechanism of blocking microbial infection by topical application of an anti-microbial peptide constitutes a major advance in overcoming the urgent problem of increased antibiotic resistance.

Plant transformation

Plant derived therapeutic proteins offer a new strategy for immunising against disease, which is a viable and competitive alternative to the use of mammalian cells and bacteria. Vegetatively propagated perennial crops that are consumed fresh, such as apples and strawberries offer an ideal subject for expression of such proteins. This project, co-funded by HDC and the East Malling Trust for Horticulture Research, aims to express a novel anti-microbial protein in plants, the SA I/II antigen of the oral pathogen *S .mutans,* in transgenic apple. Whilst this proposal focuses on *S. mutans,* it is clear that this approach is more generic, in that a number of serious human pathogens have already been identified which may be treated by application of a receptor blocking strategy, for example *Helicobacter pylori,* which causes peptic ulcers, gastric cancer and MALT lymphomas.

Objectives

- To produce new gene constructs, initially using a 35S promoter to drive expression of the SA I/II gene, for transformation
- To produce transgenic plants of apple (cv Greensleeves) expressing the SA I/II gene
- Molecular characterisation of any transgenic plants produced

Results & Discussion

The portion of the SA I/II gene encoding amino-acid residues 39-1538 was amplified by PCR incorporating start and stop codons in the primer sequences to ensure translation of transcribed mRNAs. Each PCR product was ligated into pET15b, downstream of a 'His-Tag' sequence, encoding a cleavable peptide tag that will facilitate subsequent protein purification. Restriction analysis to confirm correct fragment insertation has been carried out, and junctions with T7 promoter and terminator primers have been sequenced. The fragment was then ligated into a pJIT163 35S (constitutive promoter and terminator) cassette, in order to attach regulatory elements. The 35S cassette plus insert was then cut from pJIT163, and transferred to pGreen0029 (binary plant transformation vector) containing the *nptII* marker gene and transformed into *Agrobacterium* (LBA4404).

Two major transformation experiments were carried out with apple (cv Greensleeves). A large number of shoots were obtained, although most of these did not survive the antibiotic selection process. We now have one confirmed transgenic line of apple, and a further four putative transgenic lines. Some difficulty has been experienced in maintaining these plants *in vitro*, with poor growth being recorded during some sub-culture intervals. The reasons for this are unclear, but may be related to the expression of foreign proteins. Our initial observations of the phenomenon of multiapexing in the cultures seems to now be under control. Whilst the lines are maintained and bulked at East Malling, preliminary molecular analysis has been undertaken by our colleagues at Guy's King's and St Thomas' School of Medicine and Dentistry, King's College, London. PCR analysis showed that in one line, the entire SA I/II gene had been incorporated. Evidence from the other four lines was inconclusive, and further analyses are planned for near future. In addition, all lines will be subjected to southern analysis by Professor David James. The results from these analyses are expected in the next few months.

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