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Low-pollen-allergen ryegrasses: towards a continent free of hay fever?

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Abstract

The ryegrasses *(Lolium* spp.) are the major grass species sown for forage and amenity use in temperate areas of the world. Pollen of ryegrass is a widespread source of airborne allergens and is a major cause of hay fever and seasonal allergic asthma, which affect approximately 25% of the population in cool temperate climates. The main allergens of ryegrass pollen are the proteins Lol p 1 (35 kDa) and Lol p 2 (11 kDa). Lol p 1 and Lol p 2 belong to two major classes of grass pollen allergens to which over 90% of pollen-allergic patients are sensitive. In spite of being conserved in many plant species, the functional *in planta* role of these pollen allergen proteins remains largely unknown. We have generated and analysed transgenic plants with reduced levels of the main pollen allergens, Lol p 1 and Lol p 2 in the most important worldwide cultivated ryegrass species, *L. perenne* (perennial ryegrass) and *L. multiflorum* (Italian ryegrass). These transgenic plants will allow the study of the functional *in planta* role of these pollen proteins and the determination of potential for development of hypo-allergenic ryegrass cultivars. The commercial release of cultivars of perennial ryegrass and Italian ryegrass with reduced pollen allergens offers the potential to reduce subsequently the incidence of seasonal allergic responses. The prospects of reducing the incidence of hay fever in Australia will be increased through the widespread use of low-allergen ryegrasses.

Keywords: allergy; down-regulation; *Lolium multiflorum*; *Lolium perenne*; pollen allergens; transgenic ryegrass

Introduction

Temperate grasslands support the production of 80% of the world's cow milk and 70% of the world's beef and veal (Wilkins and Humphreys 2003). Most of this grassland has been sown by man, and of the grass species sown the ryegrasses [perennial ryegrass (*Lolium perenne* L.), Italian ryegrass (*Lolium multiflorum* Lam.)] and their interspecific F1 hybrid are the most commonly sown species in Europe,

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New Zealand, Japan, South Africa, South America and Australia. Perennial ryegrass is the dominant species both in terms of area sown and seed sales (Burgon et al. 1997). Perennial ryegrass has also been effectively domesticated as a turfgrass (Thorogood 2003) and forms the major component of most sports and amenity grasslands. The use of perennial ryegrass as an agricultural and recreational species brings it into close contact with major population centres in temperate regions of the world. These situations, in which the allergenic proteins that are present in perennial-ryegrass pollen lead the species to be one of the major causes of the seasonal allergic asthma and hay fever, constitute a major health problem for an estimated 25% of the population in cool-temperate regions (Ong et al. 1993; Tamborini et al. 1995). This paper reviews the possibility of using molecular-genetic techniques to down-regulate the expression of allergenic proteins in ryegrass pollen and thereby lead to the large-scale reduction in hay fever and seasonal allergic asthma, using Australian agriculture as a model.

Importance and distribution of ryegrasses in Australia

Perennial ryegrass is the most commonly sown pasture grass in those temperate regions of Australia that receive greater than 550 mm total annual precipitation (Figure 1). The area sown to perennial ryegrass in Australia is estimated at more than 6 million ha (Cunningham et al. 1994), with a potential area of more than 14 million ha. Perennial ryegrass is most commonly sown in conjunction with a legume species and year-round grazing of these pastures forms the basis of meat, milk and fibre production systems in the higher-rainfall zones of temperate Australia. These agricultural areas are largely along the eastern seaboard of Australia and surround the two largest cities (Sydney and Melbourne) and most of Australia's population. Perennial ryegrass is also sown extensively in amenity grasslands (including home lawns) and sports fields in these areas, many of which are allowed to flower during summer.

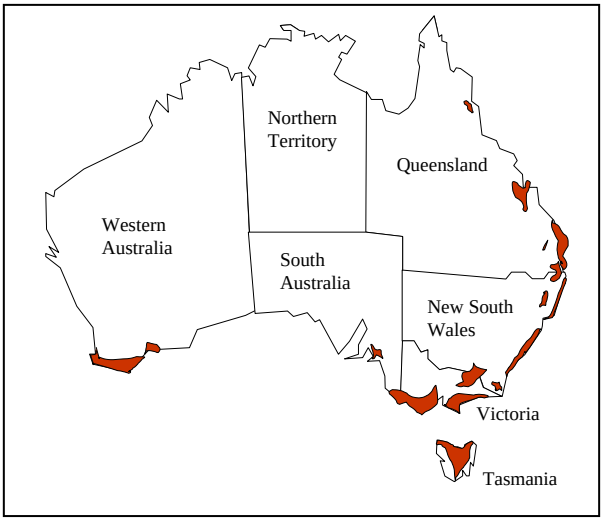

Figure 1. Distribution of perennial ryegrass pasture in Australia

Allergenic properties of ryegrass pollen

Ryegrasses (*Lolium* spp.) are the dominant source of allergenic pollen because of their wide distribution and abundant production of airborne pollen during flowering (Smart, Tuddenham and Knox 1979). A range of allergens have been identified in ryegrass pollen (Singh et al. 1991; Ong et al. 1993; Smith et al. 1994). In perennial-ryegrass pollen, at least 17 allergenic proteins ranging in size from 12 to 89 kDa have been detected (Ford and Baldo 1986; Singh et al. 1991; Sidoli et al. 1993; Knox and Suphioglu 1996).

Two different proteins designated Lol p 1 and Lol p 2 have been identified as the main allergenic determinants in ryegrass pollen (King et al. 1995). These allergens exist in multiple isoallergenic forms representing variants that appear to be immunologically indistinguishable (Johnson and Marsh 1965). Lol p 1 is the major ryegrass pollen allergen to which 95% of patients showed increased levels of IgE antibodies in their sera (Kahn and Marsh 1986). The natural allergen is an acidic, glycosylated 35 kDa monomeric protein containing intramolecular disulfide bridges (Singh et al. 1991; Johnson and Marsh 1965). Lol p 1 exists in multiple isoforms of the same size, with polymorphisms appearing in at least eight positions in the amino-acid sequence (Perez et al. 1990; Griffith et al. 1991). Lol p 1 contains a 29-amino-acid IgE-binding region at the C terminus (Esch and Klapper 1989a,b) as well as the 7 cysteine residues strictly conserved in group-1 grass pollen allergens (Singh and Bhalla 2003), and has been localized to the cytosol of the pollen grain (Singh et al. 1991).

Lol p 2 is the second most important ryegrass pollen allergen to which 45% of grass-pollen-allergic patients are reactive (Freidhoff et al. 1986). Lol p 2 is an acidic protein and is present in at least two isoforms, Lol p 2A and Lol p 2B (Sidoli et al. 1993). Lol p 2A has been purified, sequenced and found to consist of 97 amino-acid residues (Ansari, Shenbagamurthi and Marsh 1989).

These pollen allergens trigger an immune response by cross-linking specific IgE molecules bound to the surface of mast or basophil cells, leading to the release of various inflammatory mediators that are responsible for the clinical symptoms. While the mode of interaction of these pollen proteins with the human immune system has been studied in great detail (Freidhoff et al. 1986; Bieber 1996; Stingl and Maurer 1997), the functional role *in planta* of these pollen allergens remains largely unknown (Cosgrove, Bedinger and Durachko 1997; Li and Cosgrove 2001).

Down-regulation of pollen allergens

The enabling technologies for the genetic modification of ryegrasses have been established (Wang et al. 1993; 1994; Spangenberg et al. 1995; Ye et al. 1997). Transgenic ryegrass plants expressing the selectable marker hygromycin phosphotransferase (*hph*) gene have been obtained by microprojectile bombardment to embryogenic suspension cells in *L. perenne* (Spangenberg et al. 1995) and *L. multiflorum* (Ye et al. 1997). This allows the generation of materials suitable for studying the functional role of the major ryegrass pollen allergens, and the ability to assess the potential for the production of hypo-allergenic ryegrass cultivars.

We have recently developed perennial-ryegrass and Italian-ryegrass plants with down-regulated expression of the pollen allergens Lol p 1 and Lol p 2. Transformation vectors containing cDNA sequences from Lol p 1 and Lol p 2 in antisense orientation and under control of the maize-pollen-specific promoter *Zm13*

(Hamilton et al. 1992) were used for the production of transgenic plants based on biolistic transformation (Spangenberg et al. 1995). Transgenic plants were developed that exhibited normal reproductive development and pollen viability with significant reductions in Lol p 1 and Lol p 2 pollen-allergen levels assessed by immunodetection using antibodies raised against the recombinant allergens as well as sera from sensitized patients. Further characterization of these plants is underway, along with evaluation of the plants under field conditions. Transgenic plants with down-regulated expression of pollen allergens will then be crossed into a range of forage and amenity backgrounds using strategies we have developed for the production of elite transgenic germplasm (Spangenberg et al. 2001).

Modelling of pollen-based geneflow in ryegrass

The development of mathematical models to predict gene flow is well advanced in selected crops for which transformation events are widely used in agricultural production (e.g. Timmons et al. 1995; Colbach, Clermont-Dauphin and Meynard 2001a,b; Senior and Dale 2002). However, knowledge of the extent of pollen and gene flow in wind-pollinated grasses is not as well developed. Spore-traps systems to measure pollen dispersal, and hence predict gene flow, have been used in the UK for perennial ryegrass (Giddings, Sackville-Hamilton and Hayward 1997a,b; Giddings 2000). Despite the paucity of readily measured phenotypic traits suitable to measure gene flow in outcrossing forage species, studies have been performed based on the use of red pigmentation in the tiller base of perennial ryegrass (Griffiths 1950) and more recently the combination of a phenotypic trait (fertility in tetraploid perennial ryegrass) and SSR molecular markers (Cunliffe et al. 2004). The measurement of gene flow allows not only the amount of pollen to be measured but also to track its source more accurately. These studies have shown that pollen dispersal and associated gene flow follow a similar pattern that is mathematically described by a leptokurtic, negative exponential or Weibull distribution, with the bulk of pollen being deposited close to the source. In perennial ryegrass most pollen is deposited between 30 and 50 m of the source plant, with an asymptote at around 35 m from the source plants under Australian conditions (Cunliffe et al. 2004) (Figure 2). However, climatic factors such

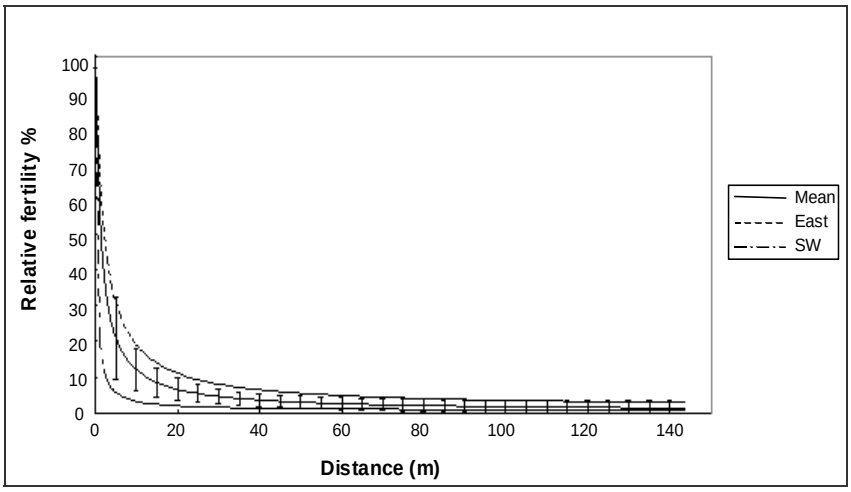

Figure 2. Fitted models for extremes of gene flow (low – southwest, high – east) and mean gene flow for all directions represented by relative fertility percent adjusted for seed viability (RF). Error bars represent 1 standard deviation based upon all measured directions

as wind speed and direction, temperature and relative humidity (Giddings, Sackville-Hamilton and Hayward 1997a,b) may interact with plant-specific factors such as height and duration of pollen shedding (Okubo and Levin 1989) to determine the exact gene flow in a given environment.

Prospects for significant reduction of hay fever

The success of low-allergen perennial-ryegrass cultivars in reducing the incidence of hay fever and seasonal allergic asthma in Australia is likely to be enhanced by a few key factors. The first major likely area of impact is in the use of low-allergen cultivars for turf. While many amenity grasslands are managed with frequent mowing to reduce the incidence of flowering, particularly those used for high-quality sports fields, there are large areas of amenity grasslands in Australian urban areas that are allowed to flower as they are mown infrequently. These include public recreation areas, roadside verges and home lawns, and represent major areas of exposure to ryegrass pollen. It is also likely that the low-pollen-allergenicity trait would provide a significant marketing advantage for amenity perennial-ryegrass cultivars.

Another relevant factor is that temperate grass-seed production in Australia is concentrated in two regions in Victoria close to the major cities of Melbourne and Geelong and several other minor rural cities. These seed-production fields represent major sources of ryegrass pollen, as the ryegrass crops here are managed to increase flowering. These areas are regularly resown as new cultivars come on to the market, so low-allergen cultivars could rapidly replace existing cultivars.

The rate at which low-allergen cultivars may replace existing cultivars in the intensive areas of perennial-ryegrass pastures in Australia can be estimated based on pasture resowing rates. These rates vary from between 5% in the driest margins of the area sown to ryegrass to greater than 20% in more intensively managed areas (Schroder and Coffey 1993). At a resowing rate of 20% it would be predicted that it would take 5 years to replace all allergenic pastures on a farm. Most of the areas with high rates of resowing are located near the major population centres. Surveys of the density of perennial-ryegrass plants in old pastures in the lower-rainfall areas of the region where ryegrass is sown have shown that very few ryegrass plants remain in some environments, so these paddocks have a large area but are likely to contribute less to the overall pollen load reaching major population centres than the intensively managed agricultural areas closer to the cities. So while more comprehensive data are required to fully model the rate of impact of low-allergen ryegrasses on hay-fever incidence in Australia, it is likely that the strategic use of low-allergen cultivars will have an immediate impact for intensively managed agricultural areas around main urban centres.

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