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Molecular features of grass allergens and development of biotechnological approaches for allergy prevention

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ABSTRACT

Allergic diseases are characterized by elevated allergen-specific IgE and excessive inflammatory cell responses. Among the reported plant allergens, grass pollen and grain allergens, derived from agriculturally important members of the Poaceae family such as rice, wheat and barley, are the most dominant and difficult to prevent. Although many allergen homologs have been predicted from species such as wheat and timothy grass, fundamental aspects such as the evolution and function of plant pollen allergens remain largely unclear. With the development of genetic engineering and genomics, more primary sequences, functions and structures of plant allergens have been uncovered, and molecular component-based allergen-specific immunotherapies are being developed. In this review, we aim to provide an update on (i) the distribution and importance of pollen and grain allergens of the Poaceae family, (ii) the origin and evolution, and functional aspects of plant pollen allergens, (iii) developments of allergen-specific immunotherapy for pollen allergy using biotechnology and (iv) development of less allergenic plants using gene engineering techniques. We also discuss future trends in revealing fundamental aspects of grass pollen allergens and possible biotechnological approaches to reduce the amount of pollen allergens in grasses.

1. Introduction

Allergies are global diseases that affect a worldwide population. Among the allergic diseases, hay fever is a widespread allergic upper respiratory condition triggered by airborne pollen allergens that activate an Immunoglobulin E (IgE) response, leading to immediate release of inflammatory mediators such as histamines. Pollen allergen exposure can also exacerbate asthma in susceptible individuals (Aalberse, 2000; Bousquet et al., 2008; Davies, 2014; Greiner et al., 2011; Scala et al., 2010). Allergic diseases affect up to 20–30% of the world population, including children as young as 3, and are associated with disrupted sleep, impaired work performance and lower life quality (Andersson and Lidholm, 2003; Bousquet et al., 2008; Dykewicz and Hamilos, 2010; Suphioglu et al., 1992; Wheatley and Togias, 2015). The severity of allergic rhinitis varies between locations and seasons due to environmental effects and pollen production time (Beggs et al., 2015; Buters et al., 2015; Davies et al., 2012; Dykewicz and Hamilos, 2010; Nony et al., 2015). Genetic and environmental variations between individual plants, as well as differences between allergen

proteins in various plants species, particularly grasses (Poaceae family), impact allergen exposure, sensitivity to allergens and symptoms of patients (Aiubi et al., 2015; Amardip et al., 2014; Buters et al., 2015; Y. Chen et al., 2016; Davies, 2014).

Allergic sensitization to aeroallergens frequently leads to the production of allergen-specific IgE antibodies in susceptible people (Moneret-Vautrin, 1997; Moneret-Vautrin and Kanny, 2007). IgE typically recognises motifs or conformational epitope structures on the surface of allergen proteins, which contain diverse and complex motifs, making it difficult to attribute IgE binding to any single linear peptide sequence (Adachi et al., 1993). Upon secondary exposure to allergens in sensitized individuals, IgE-mediated an immediate allergic reaction, prompting symptoms ranging from itching of the eyes, sneezing, and rashes, to severe anaphylaxis (Ramesh, 2008). Allergen abundance can be influenced by the environment and genetic predisposition of the source organism, and severity of symptoms can also be influenced by the genetic predisposition of the patient (Campbell and Mehr, 2015; Y. Chen et al., 2016; Fuhrmann et al., 2016). Allergens can be present in a variety of animal byproducts, cereal grains, and pollen

Abbreviations: AIT, allergen-specific immunotherapy; IgE, Immunoglobulin E; IgG, Immunoglobulin G; RNAi, RNA interference

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grains (Y. Chen et al., 2016; Sandrini et al., 2015). Food allergens have been extensively reviewed, especially in the context of common or severe allergies to dairy and nuts that cause gastrointestinal or systemic anaphylactic reactions and/or atopic dermatitis (Campbell and Mehr, 2015; Patel and Volcheck, 2015; Pomés, 2008; Skypala and Vlieg-Boerstra, 2014).

Grasses comprise one of the largest angiosperm families and contain many cultivated and widely distributed species that produce allergenic pollen and grains. Several reviews summarized respiratory diseases caused by grass, weed or tree pollen allergens (Andersson and Lidholm, 2003; Davies, 2014). In this review we focus on our understanding of the evolutionary events, common motifs and function of putative allergens in grass pollens, as well as efforts to alleviate allergies using biotechnological tools, and provide future research perspectives.

2. Global distribution of allergy-inducing grasses

Poaceae species are globally distributed and are the source of many pollen allergens. D'Amato et al. (2007) reported that airborne pollens from trees, weeds and grasses are associated with allergies across Europe. Smith et al. (2014) also proposed that the dominant species producing airborne pollens are grasses. Likewise, along with pollens of the ever-greens, grass pollens are dominant in the air in Calcutta, India (Mandal et al., 2008). In Lebanon, 17.75% of the allergy patients were allergic to grass pollen (Irani et al., 2013). Clinically important allergy-inducing grass species have been recorded across the USA (Fuhrmann et al., 2016; White and Bernstein, 2003). Pollen and grain allergies have been well documented among both adults and children on the Australian continent as pollen allergies are more prevalent in Australia than other countries, and airborne grass pollen is a major contributor to allergic rhinitis in Australia and New Zealand (Asher et al., 2006; Australian Institute of Health and Welfare, 2011; Davies et al., 2015; Robertson et al., 1998; Woods et al., 2002) (Fig. 1). In Australia, the levels of airborne grass pollens varies greatly between location and climate (Beggs et al., 2015) and the distribution of grass types varies with latitude (Davies et al., 2015; Medek et al., 2016). Bermuda grass and Bahia grass are among the major pollen contributors to allergic disease in subtropical Queensland, and Ryegrass is one of the major contributors in temperate Melbourne (Davies et al., 2011, 2012).

The Poaceae family, which contains approximately 780 genera and around 12,000 species, includes many important grain-producing agricultural species such as rice (*Oryza sativa*), wheat (*Triticum* spp.) barley (*Hordeum vulgare*), and maize (*Zea mays*). In Poaceae, allergen proteins are often found in pollen or grains. In higher plants, pollen grains are produced via meiosis and mitosis within the male organ stamen, which contains the anther and supporting filament (Fig. 2) (Shi et al., 2015; D.S. Zhang et al., 2010; Zhang et al., 2013). Each pollen produced within the stamen contains two sperm cells and one vegetative cell and is enclosed by specialised pollen walls. Pollen grains fertilise ovules, which later develop into seeds and grains. Cereal grains are small, hard, and dry seeds and are a major global food source that contains a substantial amount of starch, a type of carbohydrate that provides dietary energy and nutrition (H. Zhang et al., 2010; Zhang et al., 2013). Grass pollens contain many proteins, only some of which are allergenic and cause an IgE response (Abou Chakra et al., 2012; Andersson and Lidholm, 2003). Currently at least 20 grass species such as timothy grass (*Phleum pratense*) and wheat have been reported to produce pollen proteins associated with the triggering of an IgE response, and the majority of these species are prevalent in temperate climates (Andersson and Lidholm, 2003). Rice, maize and wheat are crops that are grown and consumed on every continent excluding Antarctica. Therefore investigation of pollen allergens in agricultural Poaceae species is of great importance to the prevention of pollen allergies.

3. Allergens in Poaceae species

Allergen proteins are found in both pollens and grains and the number of allergen families varies between species. Wheat for example, contains up to 27 allergen families as characterized by the IUIS/WHO guidelines (Radauer et al., 2012, 2014). A number of allergen families are reported in grass pollens and/or grains, including β -expansin proteins Phl p 1 from timothy grass and Zea m 1 from maize, the lipid-transfer protein (LTP) Zea m 14 from maize, and the gliadin proteins Tri a 19, Tri a 20 and Tri a 21 from wheat (Table 1) (Anderson et al., 1989; Golias et al., 2013; Lehto et al., 2003; Matsuo et al., 2005; Pasini et al., 2002; Pastorello et al., 2013; Russell et al., 2008; Staiger et al., 1993; Trcka et al., 2012). Expansins are cell wall-loosening proteins that at least partially mediate the pH-dependent cell wall extension and cell growth. Expansins include two families, α -expansins and β -expansins, and are reported to be involved in modulating a variety of plant developmental events besides cell expansion, including organ morphogenesis, softening of fruits, and grass pollen tube growth (Artzi et al., 2016; Cho and Cosgrove, 2002; Grobe et al., 1999; Li et al., 2003; Marowa et al., 2016; Sampedro and Cosgrove, 2005; Valdivia et al., 2003, 2009; Wu et al., 2001; Yoo et al., 2003). Plant LTPs belong to a small and abundant lipid-binding protein group that exchange lipids between membranes, and have functions in various processes such as anther development and pollen formation (D.S. Zhang et al., 2010; Zhang et al., 2008). Gluten is a mixture of hundreds of related but distinct wheat storage proteins, mainly gliadin and glutenin (Anderson et al., 1989; Gil-Humanes et al., 2008; Gil-Humanes et al., 2011; Shewry et al., 2002; Weegels et al., 1996). Gliadin proteins in wheat grains promote dough elasticity and strength, and can also trigger an IgE response in allergy patients (Matsuo et al., 2005; Shewry et al., 2002). Similar storage proteins in other species, such as secalin from rye, hordein from barley, and avenin from oats, have also been collectively called gluten (Biesiekierski, 2017). Due to the prevalence of gluten in wheat and other grains, patients need to avoid wheat-based foods. However, because it is difficult and expensive to find appropriate dietary alternatives to allergenic grains, especially in countries where grain-based diets are the primary staple, it is important to understand the fundamental aspects of grass allergens and develop tools to alleviate food allergies.

Several studies in Poaceae species have identified allergen genes based on investigations using transcriptomics and proteomics as well as sequence comparison or IgE binding assays (Campbell et al., 2015; Golias et al., 2013; Li et al., 2012; Sekhar et al., 2015). Some putative allergens have been identified in DNA or transcriptome databases based on sequence similarity, but currently little is known about the specific structural or functional properties of proteins encoded by these sequences. Moreover, in many cases the expression of such protein sequences in relevant plant tissue and the capacity of these putative allergens to bind IgE or to elicit an allergic reaction de novo have not been demonstrated. Major groups of grass allergens discussed in this review include the α -amylase/trypsin inhibitors, β -expansin-like proteins expressed in pollen, and profilins expressed in both pollen and grain (Aalberse, 2000; Radauer et al., 2008, 2012, 2014).

4. Structure and conserved features of grass allergens

Putative allergen proteins can be predicted based on sequence homology compared with previously known allergens or known IgE-binding epitopes, but must be confirmed through immunological tests such as serological IgE assays or skin prick tests (Radauer et al., 2012, 2014). IgE binding epitopes can be comprised of continuous linear or discontinuous stretches of amino acids, but these motifs can be highly varied in amino acid sequences (Matsuo et al., 2015). Understanding how these allergen encoding genes evolved may be helpful in revealing allergenic properties and for designing future allergy prevention strategies.

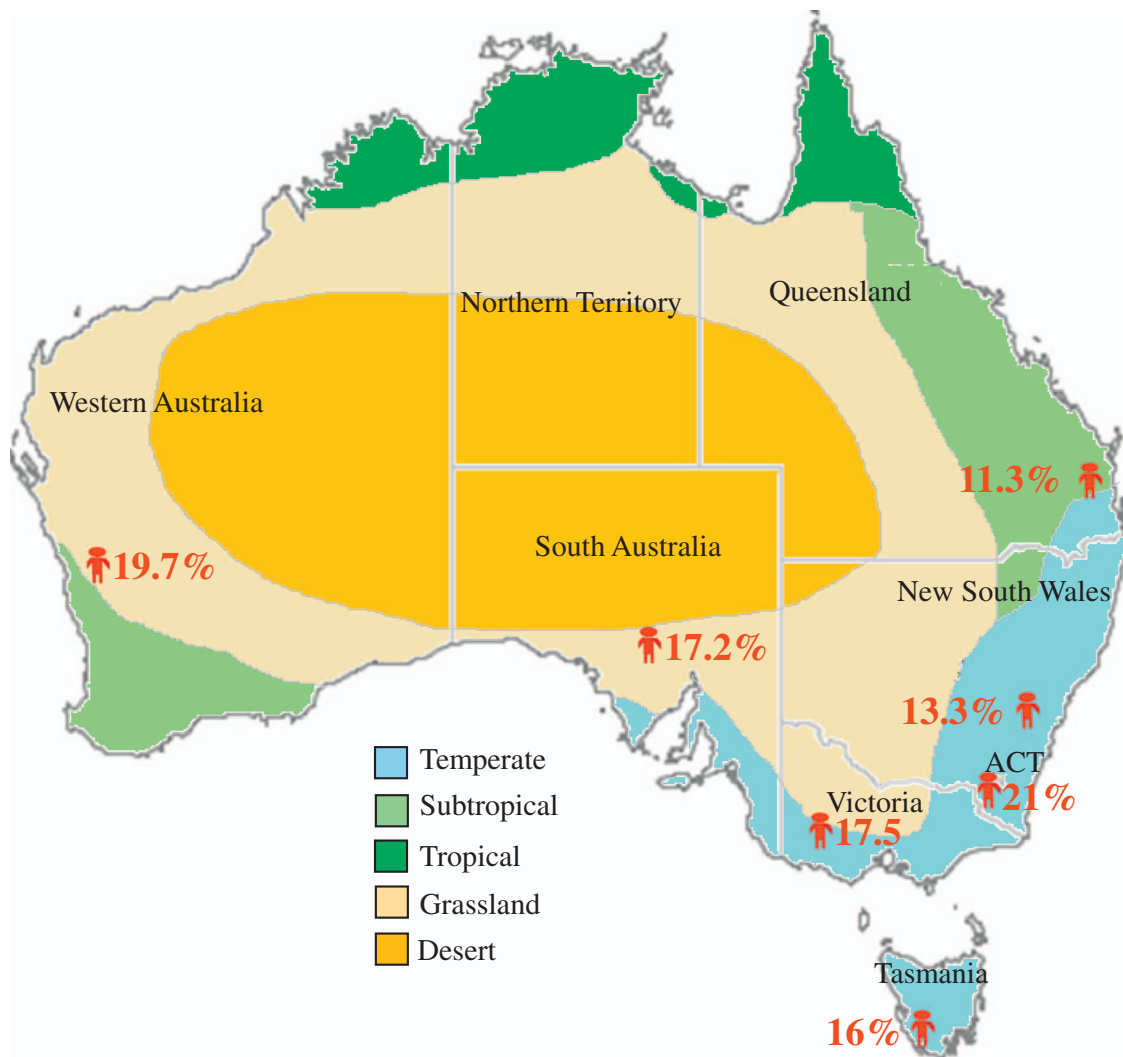


Fig. 1. Climate and prevalence of hay fever in Australia. Approximate percentage of state population with hay fever symptoms during peak season (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) Adapted from [Australian Institute of Health and Welfare \(2011\)](#).

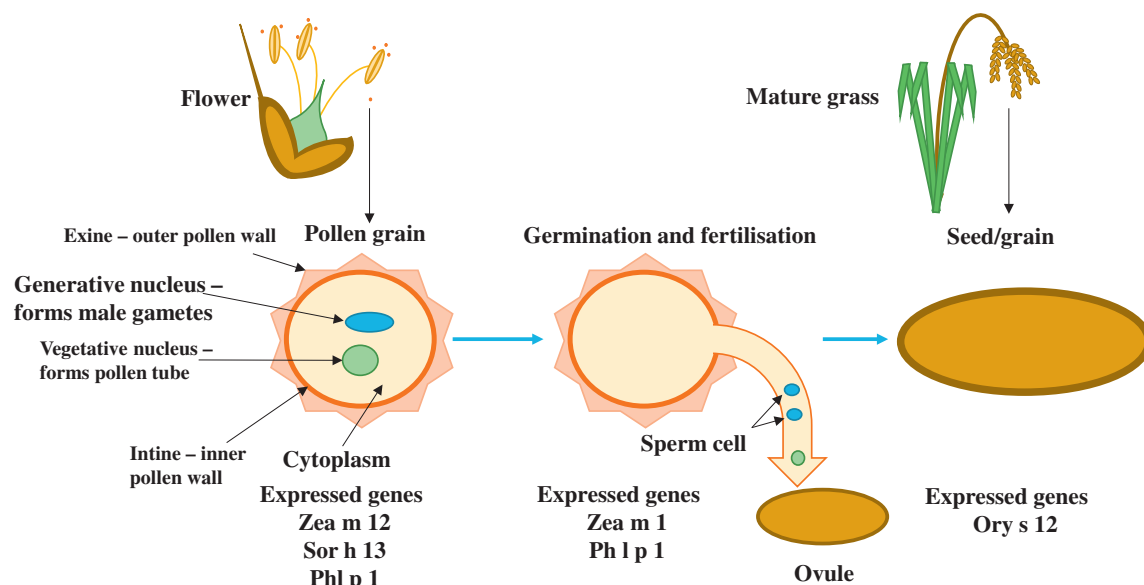


Fig. 2. Pollen germination and fertilisation.

Table 1
Putative allergens characterized by WHO/IUIS guidelines – adapted from [M. L. Chen et al. \(2016\)](#). Allergens from agricultural Poaceae species and their putative functions. Biological functions that have not been experimentally confirmed are marked with an asterisk.

Species	Putative gene family/ homolog	Allergen name	Biological function	Reference
<i>Hordeum vulgare</i> (barley) <i>Oryza sativa</i> (rice)	Profilin	Hor v 12	Actin-binding protein*	Astwood et al. (1995)
	β-Expansin	Ory s 1	Promotes cell wall expansion*	Xu et al. (1995)
	Profilin	Ory s 12	Actin-binding protein*	Ye et al. (2001)
<i>Phleum pratense</i> (timothy grass)	Pollen Ole e 1 allergen (10)	Phl p 11	Accumulates in pollen tube cell walls, possibly to aid in germination control or pollen tube emergence	de Dios Alché et al. (2004), Marknell DeWitt et al. (2002)
	Polygalacturonase	Phl p 13	Pollen expressed polygalacturonase has a role in pollen maturation and pollen tube growth*	Suck et al. (2000)
<i>Sorghum halepense</i> (johnson grass)	Profilin	Phl p 12	Actin-binding protein*	Valenta et al. (1994)
	β-Expansin	Phl p 1	Promotes cell wall expansion*	Petersen et al. (1995), Valenta et al. (1992)
	Polygalacturonase	Sor h 13	Pollen expressed polygalacturonase has a role in pollen maturation and pollen tube growth *	Campbell et al. (2015)
<i>Triticum aestivum</i> (wheat)	β-Expansin	Sor h 1	Promotes cell wall expansion*	Bashir et al. (2013), Campbell et al. (2015)
	Gliadin	Tri a 19, Tri a 20, Tri a 21	Nutrient reservoir activity*	Matsuo et al. (2005), Sander et al. (2011)
	Glutenin	Tri a 26	Accumulates in the endosperm and forms a protein matrix. This matrix may provide carbon, nitrogen and sulphur storage and aid in seedling growth.	Anderson et al. (1989), Shewry et al. (2002), Weegels et al. (1996)
<i>Zea mays</i> (maize)	Lipid transfer protein	Tri a 14	Lipid transportation*	Sander et al. (2011)
	Profilin	Tri a 12	Actin-binding protein*	Rihs et al. (1994)
	Profilin	Tri a 27	Provides enzymatic oxygen reduction in multiple tissues. This prevents oxidative stress in both reproductive and vegetative tissue, and can also move between cells, allowing them to indirectly control multiple processes	Maeda et al. (2006), Meng et al. (2010), Sander et al. (2011)
	α-Amylase inhibitor	Tri a 28, Tri a 29, Tri a 30, Tri a 40	Directly inhibits α-amylase from insect salivato prevent amylase damage to leaves	García-Maroto et al. (1990), Lullien et al. (1991), Sander et al. (2011)
<i>Zea mays</i> (maize)	Polygalacturonase	Zea m 13	Pollen expressed polygalacturonase has a role in pollen maturation and pollen tube growth	Allen and Lonsdale (1992), Petersen et al. (2001)
	Lipid transfer protein	Zea m 14	Lipid transportation*	Pastorello et al. (2000)
	Profilin	Zea m 12	Actin-binding protein*	Gibbon et al. (1998), Staiger et al. (1993)
	β-Expansin	Zea m 1	Induces irreversible cell wall expansion and promotes pollen tube growth and penetration of ovule by pollen tube. Ovule penetration influences pollen-pollen competition. Expansins with cellulose-binding-like domains are specific to monocots.	Broadwater et al. (1993), Cosgrove et al. (1997), Sampedro and Cosgrove (2005), Sampedro et al. (2015), Valdivia et al. (2007)

Understanding the structure of allergens in the Poaceae family will help to uncover common features of allergens and identify targets for manipulation by gene technology. Particularly, allergens have varied high-order structures between protein families but conserved tertiary structure between homologs, raising the question of what causes a protein to have allergenic properties (Andersson and Lidholm, 2003; Petersen et al., 2001). One explanation of IgE cross-reactivity between grass allergens is that cross-reactivity may be due to structural similarities; thus, understanding structural differences and similarities among grass allergens will also clarify the evolutionary events of allergen genes in grasses (Hrabina et al., 2008; Marth et al., 2004). As more grass genome sequences and gene expression data become available, bioinformatics research promises to uncover more candidate allergenic proteins in grasses.

Different allergen families differ in their protein tertiary structures. For example, there are four main structures in the 40 allergens that represent all types of allergen structures: α -helices, α -helix and β -sheet associated structures, structures of α -helices and β -sheets that are not associated, and anti-parallel β -sheets (Aalberse, 2000). The varied structures of allergens suggest that allergens may not have a specific common domain, but that any antigen can be allergenic if they have an ability to stimulate IgE and allergenic responses (Aalberse, 2000). Potentially, IgE recognises smaller structures of few amino acids in length. Two profilins, Hev b 8 from rubber tree and Zea m 12 from maize, were shown to contain a side loop primarily responsible for most IgE binding activity (Mares-Mejia et al., 2016). Furthermore, IgE binding was highest when Hev b 8 was in monomeric form, suggesting that some IgE epitopes are no longer exposed when Hev b 8 is in the dimeric form (Mares-Mejia et al., 2016). Therefore, understanding how structure relates to IgE binding avidity is essential for producing novel recombinant proteins for therapy.

Interestingly, Poaceae allergens share conserved cysteine between allergen family homologs in different taxa. Ten conserved cysteine residues in allergenic α -amylase/trypsin inhibitors were identified some allergens from rice, wheat and barley (Nakamura and Matsuda, 1996; Nakase et al., 1998). Likewise, Wang et al. (2014) revealed two cysteine rich motifs in allergenic α -amylase/trypsin inhibitors but they were not present in non-allergenic homologs. Phylogenetic analysis showed that these α -amylase/trypsin inhibitor genes in rice were grouped in the same clade, suggesting that the cysteine residues are preferentially retained in the same evolutionary lineage (Wang et al., 2014). Another study analysed the structure of multiple wheat flour allergens and showed conservation of cysteine residues and disulphide bridges, suggesting that these structural features may play an important role in the function of allergens (Wang et al., 2013). Furthermore, cysteine residues are found in α -amylase/trypsin proteins, profilin proteins and β -expansin grass allergens (Fig. 4). However, how these residues are linked to protein function and allergenicity, and the functional consequences of disrupting disulphide bridges remain unclear.

5. α -Amylase inhibitors and trypsin inhibitors

α -Amylase is an enzyme existing in many organisms that is able to hydrolyse α -bonds of large, α -linked polysaccharides including starch and glycogen, forming glucose and maltose. Trypsin inhibitors are a type of serine protease inhibitor with the ability to reduce the biological activity of trypsin, an enzyme involved in the breakdown of a great variety of proteins. There have been reports that some genes encoding allergens show high homology to both α -amylase and trypsin inhibitor genes, suggesting that protein products of these genes may have multiple functions (Alvarez et al., 1995). Plant α -amylase inhibitors can inhibit the activity of digestive α -amylase in insect saliva, thus playing a protective role in plants (Franco et al., 2002; Kaur et al., 2014; Major and Constabel, 2008; Mehrabadi et al., 2012; Pytelkova et al., 2009). Trypsin inhibitors are also involved in plant defence

through protein degradation (Rogozhin et al., 2012). The expression of α -amylase/trypsin inhibitors is frequently seen in seeds of cereals such as barley and wheat and the α -amylase/trypsin inhibitor genes may have arisen during evolution following grass diversification (Adachi et al., 1993; Iulek et al., 2000; Wang et al., 2014). Notably, sequence-based prediction of α -amylase inhibitor- and trypsin inhibitor- types of allergens may not be accurate. For instance, in rye, α -amylase inhibitors also acted as a trypsin inhibitor proteins, but Iulek et al. (2000) showed that homologs in wheat and barley were unable to inhibit trypsin like in rye. Rice contains at least 7 α -amylase/trypsin inhibitor genes, but their function is currently unknown (Wang et al., 2014). As grain expressed allergens, α -amylase and trypsin inhibitor proteins are likely to be denatured during cooking, losing their allergenicity easily.

6. Profilins

Present in both monocots and dicots, profilins control the growth of actin microfilaments and cytoskeleton restructuring (Gibbon et al., 1998; Sun et al., 2013; Wang et al., 2006). Profilins have sequence homologs that are expressed in vegetative tissues and/or reproductive organs, but the function of the putative allergenic profilins remains unknown. In *Arabidopsis thaliana* (Arabidopsis), profilins were shown to be partially redundant in function, but also have specific expression pattern and functions in determining leaf morphology and root growth (Müssar et al., 2015). In maize, there may be at least two functionally distinct profilin groups, one group inhibiting the hydrolysis of phospholipids and the other group involved in the dynamics of actin filament (Kovar et al., 2000). A proteomic study found the maize profilin-like putative allergen, Zea m 12, in the pollen coat, suggesting that this protein may play a specific role in this tissue (Wu et al., 2015). However, not all profilins are allergenic. Very low IgE reactivity was found for Zea m 12, indicating that it may not be a dominant allergen in grasses (Petersen et al., 2006; Radauer and Breiteneder, 2006). There is fairly low sequence similarity between allergenic and non-allergenic profilins (30–40%), but the allergenic properties have not been well characterized in grasses and the relationship between their function and allergenicity remains unclear (Santos and Van Ree, 2011).

7. β -Expansins

β -Expansins are cell wall expansion proteins expressed in multiple plant tissues. This family of allergens includes the Group 1 grass specific pollen allergens, which are the major allergens in grass pollen that account for the majority of IgE binding in patient sera and to which there is currently no prevention (Hirano et al., 2013). Although Group 1 allergens are β -expansin proteins in sequence, they exist in multiple isoforms within a single species and may have multiple functions (Cosgrove et al., 1997; Sampedro and Cosgrove, 2005). Expansins promote fruit softening in fruit-producing plants and root hair initiation in Arabidopsis, but these expansins may differ in function from grass specific β -expansin proteins (Cho and Cosgrove, 2002; Kalamaki et al., 2003; Yoo et al., 2003). Many allergenic β -expansin genes are expressed only in pollen and are involved in cell-wall loosening to promote pollen tube growth towards the ovary (Valdivia et al., 2009). In contrast, non-allergenic β -expansin genes are expressed in vegetative tissue, suggesting that allergenic β -expansin genes may have obtained diversified functions during evolution (Lee and Kende, 2001; Sampedro and Cosgrove, 2005; Sampedro et al., 2015; Schipper et al., 2002; Valdivia et al., 2009; Wu et al., 2001). One maize allergen, Zea m 1, was shown to play a role in pollen-pollen competition by influencing ovule access (Valdivia et al., 2007). In addition, mutations of some β -expansin allergen genes caused disrupted pollen formation, pollen dispersal and overall reproductive fitness, so β -expansin proteins likely play a crucial role in pollen development (Valdivia et al., 2009). Furthermore, Zea m 1 was also shown to have a role in pollen tube growth and ovule penetration, as mutated Zea m 1 exhibited aborted

pollen tube growth, potentially due to reduced cell wall expansion and loosening activity (Cosgrove et al., 1997; Valdivia et al., 2009). Given these findings in maize, it is likely that β -expansins in other grasses may also be involved in pollen development and pollen tube growth. To this end, it will be important to investigate the full function within the plant of β -expansin-like allergen encoding genes in other Poaceae species.

7.1. Evolution and functional prediction of pollen allergens

Recently, we performed genome-wide analysis of sequences encoding putative pollen allergens, and predicted 145 and 107 candidate pollen allergens from rice and Arabidopsis, respectively (M. L. Chen et al., 2016; Cui et al., 2012). These pollen proteins are likely involved in metabolic processes such as cell wall metabolism and may play roles in stress responses based on gene expression data (M. L. Chen et al., 2016). Genes that encode these proteins appear to have evolved from large gene families and later diversified during evolution. Furthermore, sequence analysis among 25 plant species from green alga to angiosperms suggested that about 40% of putative pollen allergenic proteins, including profilins, are conserved from lower to higher plants, while other allergens such as β -expansins seemed to be formed later in evolution. Although a high frequency of gene duplication was observed among allergen-coding genes, we predicted that these allergen-coding genes might have been selected by purifying selection during evolution. To explain the phylogenetic and evolutionary scenario of genes that encode putative pollen allergens, we proposed that many allergen gene families experienced both duplication and deletion events that resulted in highly variable degrees of conservation between species (Fig. 3). Genes originated from a putative allergen-coding gene may have experienced multiple duplications, resulting in increased copy number, and these genes may have either been maintained, deleted or modified to lose allergenicity or evolve tissue specific functions. Non-allergen encoding genes may have been duplicated and later modified to gain allergenicity, resulting in gene families that contain genes for both allergenic and non-allergenic proteins.

7.2. Allergens derived from ancient and recent duplications

Some allergen-encoding genes have been conserved in Poaceae species. A study of a putative rice allergen Ory s 2, showed a 33% sequence identity between the C-terminus of this gene and ryegrass (*Lolium perenne*) allergen genes, suggesting that conservation of allergen sequences occurs between grasses (Kerim et al., 2003). However, no immunological cross-reactivity was found between Ory s 2 and other grass allergens with similar protein structures (except wheat), suggesting that these conserved regions may not necessarily pertain to allergenicity (Kerim et al., 2003). However, another research group identified five wheat allergen cDNAs that encode proteins sharing IgE reactivity and cross-reactivity with other putative allergenic seed

proteins in grains such as barley, rye and rice (Pahr et al., 2012). The cross-reactivity of these wheat allergens and seed proteins in other grasses suggests that these proteins may contain conserved motifs stemming from a common ancestor, raising the question as to whether the putative allergenicity is structurally or functionally significant. As such, structural and functional variation of putative allergens between grasses needs to be further studied to understand why the level of conservation varies.

Bioinformatics analysis suggested that Poaceae allergen genes may have arisen both prior to and following the divergence of grasses. For instance, several β -expansin genes may have evolved before the grass species diverged, but also underwent rapid evolution following divergence (Sampedro et al., 2015). β -expansin genes can be classified into two groups; one group existed before monocots and dicots diverged and has been conserved after the divergence, and the other group arose only in monocots, suggesting that the latter group may have a grass specific function (M. L. Chen et al., 2016). The rice β -expansin Ory s 1 is a major rice pollen allergen that is also conserved in maize, wheat, barley, timothy grass, johnson grass (*Sorghum halepense*), panic grass (*Panicum hallii*) and sorghum (*Sorghum bicolor*). These homologs are varied in gene copy number in different grass species, from 2 copies in timothy grass to 12 copies in maize. These evolutionary events may provide insight into the importance of copy number in grass species and aid in gene technology and gene silencing strategies to reduce allergens in transgenic plants.

7.3. Allergen genes duplicated within one single grass species

Phylogenetic analyses of all the allergen groups in multiple species could help to further understand the evolution and possibly the role of these genes in Poaceae. Understanding the evolution of allergenic and non-allergenic homologs in one species may also reveal why some homologous genes are allergenic and others are not, and why multiple allergen genes have been evolved. Allergen genes in Poaceae species were shown to be conserved within the genome of single species. Russell et al. (2008) showed that highly homologous allergen genes in rice often lack introns and have multiple copies of the gene located near each other on DNA. For example, seven α -amylase/trypsin inhibitor allergen genes, all of which are expressed in seeds, are on chromosome 7 in rice (Wang et al., 2014). It was proposed that high copy numbers of rice pollen allergen genes were the result of an insertion of highly expressed genes that were modified to be more efficiently translated (Wang et al., 2014). M. L. Chen et al. (2016) proposed that these allergen gene families may be caused by frequent duplications, after which the allergen gene or allergenic feature of the gene has been retained. These findings raised the question of how efficient translation and gene copy number affect allergenicity and plant function.

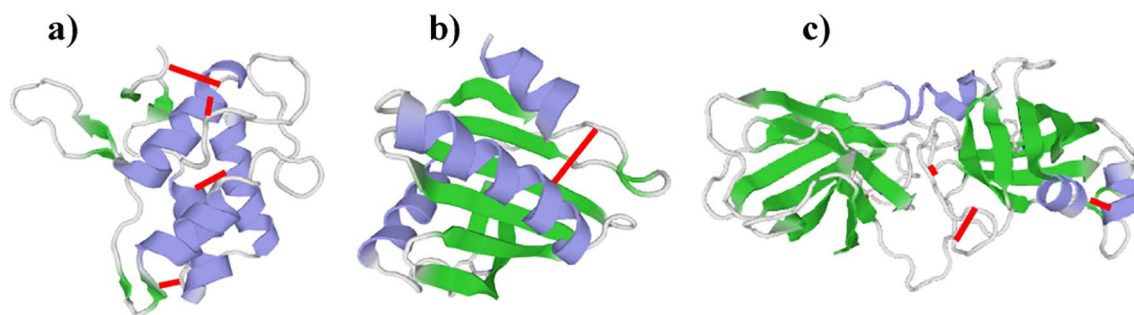


Fig. 3. Tertiary structure of Profilin, β -expansin and α -amylase/trypsin inhibitor. Tertiary structures of maize α -amylase/trypsin inhibitor (a), *Arabidopsis thaliana* profilin (b) and timothy grass β -expansin Phl p 1 (c) consist of α -helices (blue), β -sheets (green) and disulphide bridges (red bar). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Images were retrieved from SWISS-MODEL (Biasini et al., 2014).

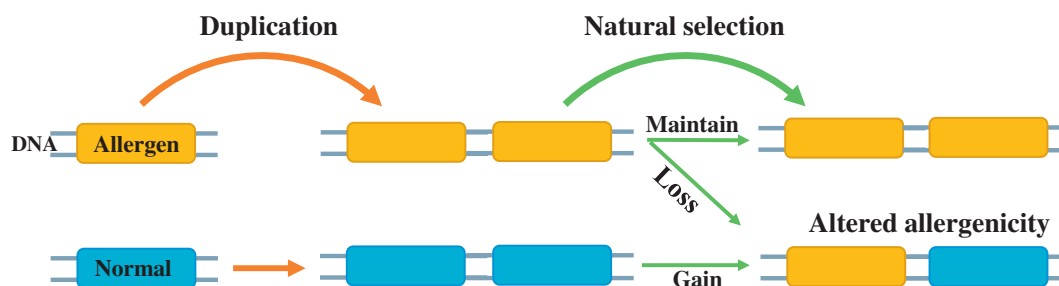


Fig. 4. Evolution of allergen genes. Allergen genes (yellow) and non-allergenic genes (blue) may experience duplications that lead to multiple gene copies. Through the course of natural selection, these copies can be maintained, lost or new allergens may be gained from non-allergenic genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Adapted from M. L. Chen et al. (2016).

8. Biotechnological approaches to prevent allergies to Poaceae allergens

Although avoiding exposure to allergens is the most common form of prevention of allergic responses, post-harvest treatment is also important for alleviating allergenic properties (Dodo et al., 2008; Ogawa et al., 2000; Yu et al., 2013). Enzymatic treatment of harvested foods can decrease the concentration of allergen proteins in multiple foods, and transgenic foods with decreased level of allergen proteins resulted in lower IgE binding, decreased severity and prevalence of allergic reaction (Le et al., 2006a,b; Nakamura and Matsuda, 1996; Wakasa et al., 2011). However, due to the durable structure of pollen grains it is difficult to reduce allergenicity caused by pollen proteins.

9. Allergen-specific immunotherapy

Allergen-specific immunotherapy (AIT) has been used clinically for over 100 years to alleviate pollen allergies, but research into improving efficacy, efficiency, safety and specificity remains an active area of research. Research advances in AIT for grass pollen focus on temperate grass species and include the use of recombinant pollen allergen molecules (Nandy et al., 2015; Patel and Volcheck, 2015). A review paper by Calderon et al. (2012) summarized the safety and efficacy of AIT for grass pollen allergy prevention. Investigations on the use of AIT against timothy grass pollen allergy include (1) employment of different routes of administration such as subcutaneous or sublingual, which impact the cellular uptake of antigens by dendritic cell subtypes or B cells, (2) processing and presentation of allergens to induce tolerance by induction of T regulatory lymphocytes, and (3) deletion of allergen-specific T helper 2 (allergy-prone) lymphocytes (Nandy et al., 2015; Rolland and O'Hehir, 1998; Sandrini et al., 2015; Valenta et al., 2016). Several new products for temperate grass pollen allergen immunotherapy are in clinical use as AIT (Cox et al., 2012; Dahl et al., 2006; Didier et al., 2009; Nelson, 2014; Senna et al., 2011; Sjölin et al., 2008).

Another promising method for relieving allergies through AIT is through modifying allergenic proteins. Focke-Tejkl et al. (2015) fused timothy grass pollen allergens with non-allergenic peptides for the production of hypoallergenic recombinant allergen derivatives. In murine models of AIT, Immunoglobulin G (IgG) antibodies were induced instead of IgE, and these antibodies could inhibit IgE-binding without causing allergic reactions, while still being able to induce immune tolerance (Focke-Tejkl and Valenta, 2012; Focke-Tejkl et al., 2015). Treatment with synthetic peptide immuno-regulatory epitopes has decreased symptoms of grass pollen allergies to both timothy grass and bermuda grass (*Cynodon dactylon*) (Ellis et al., 2014, 2015). Additionally the fusion of timothy grass pollen allergens with mannin, a plant polysaccharide, produced a hypoallergenic protein derivative that showed a significant reduction of IgE binding (Manzano et al., 2016). Furthermore, Devanaboyina et al. (2014) demonstrated that single point mutations in the predicted IgE binding sites on timothy

grass pollen allergen Phl p 3 caused a 30% decrease in IgE binding activity compared with the wild type. Meanwhile this research also showed that a wide variety of the amino acid structures could be recognised by IgE (Devanaboyina et al., 2014). Revelations of how IgE recognises allergenic motif/structures will be very useful for the development of novel AIT strategies.

Pollen allergens elicit allergic sensitization via a respiratory mucosal route. However, allergens within the grain can cause symptoms as severe as anaphylaxis when ingested. Gluten is a mixture of grain proteins, some of which are major allergens. An AIT based on small peptide sequences of gluten was able to combat celiac disease caused by wheat and rye (Anderson, 2008; Nabavi and Hoseinzadeh, 2009). Although this AIT has entered phase 1 clinical trials, an effective wheat allergen AIT has not yet become commercially available (Brown et al., 2011; Veeraraghavan et al., 2015).

AIT has been reported to cause a range of side effects, such as asthma, increased severity of allergic reactions, and anaphylactic shock (Calderon et al., 2012; Winther et al., 2006). For instance, one study on grass allergy therapy showed that about 30% of patients treated for grass allergy developed mild respiratory side effects after a three-year administration of injected allergen immunotherapy, and 4% of these patients discontinued treatment due to severe symptoms (Winther et al., 2006). Furthermore, 46% of the patients in the test group of a double-blind study of orally administered allergen therapy reported site-related side effects such as itching, compared to 4% in the placebo group (Dahl et al., 2006). Although these reactions were mostly mild and short-lived, 5 of the 316 tested patients withdrew due to continued side-effects related to the treatment (Dahl et al., 2006). It was explained that these side-effects are possibly due to variation in IgE sensitivity of individual patients to specific allergens (Calderon et al., 2012; Focke-Tejkl and Valenta, 2012). Furthermore, modified allergens with decreased IgE binding ability that were used in allergy therapies have been shown to retain immune T-cell response and induce immune tolerance (Kahlert et al., 2000). Further research into safer AITs is needed. Alternative therapies such as using transgenic hypoallergenic crops may provide a possible solution to these problems.

10. Genetic modification of plant allergen gene expression

Gene technology, which has been carried out with ryegrass and rice, provides the possibility of producing hypoallergenic plants that do not trigger an immune response. Possible methods to silence allergen genes include RNA interference (RNAi) and gene knockout approaches (Table 2). Studies in ryegrass used a pollen-specific rice promoter, *Ory s 1* promoter, to control the expression of an anti-sense sequence of a ryegrass pollen allergen Lol p 5, which resulted in hypoallergenic ryegrass that exhibited low Lol p 5-specific IgE binding (Bhalla et al., 1999; Singh and Bhalla, 2008; Takagi et al., 2005). However, small amounts of remaining allergenic proteins could still trigger an immune response (Bhalla and Singh, 2004; Bhalla et al., 1999; Singh and Bhalla,

Table 2

Comparison of biotechnological strategies for Poaceae allergen specific immunotherapy.

Species	Allergen source	Targeted allergen	Therapy	Method	Factor altered	Compared to wild type (%)	Reference
Rice	Seeds	14–16 kDa fragment	GMO plant	RNAi silencing	Transcript levels	20	Adachi et al. (1993)
	Pollen	RA17	GMO plant	RNAi silencing	Transcript levels	20–30	Tada et al. (1996)
	Unknown	AK107328, AK066092	GMO plant	RNAi silencing and gene knock out	IgE reactivity	10	Wakasa et al. (2011)
Ryegrass	Pollen	Lol p 5	GMO plant	RNAi silencing	IgE reactivity	0	Bhalla and Singh (2004)
	Pollen	Lol p 5	GMO plant	RNAi silencing	IgE reactivity	0	Bhalla and Singh (2004)
	Unknown	Lol p 1, Lol p 2	GMO plant	RNAi silencing	Protein levels, IgE reactivity	Unknown	Petrovska et al. (2004)
Timothy grass	Unknown	Lol p 5	Oral AIT		IgE reactivity	0	Takagi et al. (2005)
	Pollen	Phl p 3	AIT	Single nucleotide polymorphism	IgE reactivity	30	Devanaboyina et al. (2014)
	Pollen	Phl p 1, Phl p 2, Phl p 5, Phl p 6	AIT	Non-allergenic peptide fusion	IgE reactivity	0.5–60	Focke-Tejkl et al. (2015)
	Pollen	Unknown	AIT	Fusion of allergen protein with mannin	IgE reactivity	20–25	Manzano et al. (2016)
	Pollen	Unknown	AIT	Fusion of allergen protein with mannin	IgE reactivity	20–25	Manzano et al. (2016)

2008). Another study reported the down-regulation of the major ryegrass pollen allergens Lol p 1 and Lol p 2 by placing anti-sense Lol p 1 and Lol p 2 under the pollen-specific maize Zm13 promoter. The resultant transgenic plants showed decreased allergen levels and IgE reactivity (Petrovska et al., 2004). These studies were conducted over ten years ago, but no follow up studies have been reported on how to improve application of this technology. Technically, grass pollen and grain allergens are frequently encoded by multiple copies of genes, so a complete knock-out of the allergens is difficult. Furthermore, understanding the function of gene homologs in other grass species may aid in understanding whether modified hypoallergenic protein peptides can be used in place of wild type allergens in transgenic plants (Suphioglu et al., 1992; Valenta et al., 1991, 1992).

Development of wheat grains with reduced allergenic gliadin has been achieved through the use of RNAi (Gil-Humanes et al., 2008, 2010). Grains from these transgenic lines had reduced T-cell binding, so flour produced from these grains may be an alternative bread source for celiac patients, (Gil-Humanes et al., 2008, 2011, 2014). However, IgE binding analyses were not carried out in these studies, so it is unclear whether these transgenic lines will be suitable for patients with wheat allergy. Moreover, wheat contains other allergens that may still be present in these transgenic lines, and it is unclear whether biological function of these transgenic plants is affected.

Biotechnology has also been used to develop hypoallergenic rice lines. Transgenic plants containing allergen-specific antisense RNA showed approximately 20% of α -amylase/trypsin inhibitor allergens in grains compared with the wild type (Nakamura and Matsuda, 1996). Likewise, an RNAi approach decreased the transcript levels of α -amylase/trypsin inhibitor allergens to 20%–30% in transgenic rice pollen (Tada et al., 1996). However, there were no analyses on the immune response of patients, so it is unclear whether the decreased transcript and protein levels would be effective in reducing allergic responses. These two studies were conducted in 1996, but there has been no follow-up report on using these rice lines nor is it clear whether biological function was affected in the transgenic plants. By knocking out a rice 26 kDa allergen (GbN-1) coupled with RNAi of another two allergens, α -globulin and β -glyoxalase, transgenic plants had decreased allergen levels in grains and a dramatic decrease (to about 10%) of IgE binding activity in sera of all 15 patients in the study compared to the control sera (Wakasa et al., 2011). These studies did not report on whether silencing allergen genes disrupted plant growth or function, so a functional analysis is needed to assess the viability and performance of hypoallergenic plants.

Gene knock-out or RNAi-mediated silencing of pollen-specific allergens may lead to disrupted pollen development and fertility or an insufficient decrease in allergen levels in transgenic grasses.

Therefore, it is necessary to explore gene-site-specific modification as an alternative method to reduce the level or alter the structure of pollen-specific allergen proteins, which can potentially reduce allergic responses while maintaining normal biological development and function of the plant. Genome editing technology such as CRISPR-Cas9 has been shown to be effective in many grasses, but has not yet been used to inhibit allergen activity (Feng et al., 2016; Kim et al., 2016; Li et al., 2016; Ma et al., 2015, 2016). This method may allow allergen families to be efficiently altered or reduced in expression in order to develop hypoallergenic plant derivatives that maintain biological function.

11. Challenges and future directions

Biotechnological approaches aimed at developing faster, safer and more targeted AIT has been heavily investigated in plants, particularly for grass pollen-specific AIT (Calderon et al., 2012; Passalacqua and Canonica, 2016). Focusing on reducing the expression or mutating the allergenic genes may provide a more convenient method to overcome pollen and grain induced allergic diseases compared with the need for prolonged and frequent AIT administrations. Transgenic plants may provide a relief for field workers during harvest and offer alternative consumables to overcome grain allergies, making these plants an economically desirable product. Ethical concerns about how transgenic grass allergens affect humans and the environment may prevent commercial distribution of hypoallergenic plants, but a better understanding of the function and role of allergen genes and comparative studies of transgenic and non-transgenic plants may help to ease some of these concerns (Graf et al., 2014; Vidal et al., 2015).

With the advance of bioinformatics and functional genomics, it is possible to investigate the biological function and the role of allergens in plant development. Future work on the history and molecular function of allergens and the knowledge applicable from one species to others is also critical. The structure and IgE binding efficiency of pollen and grain allergens varies greatly between plant species and cannot yet be attributed to a sole motif or protein structure (Aalberse, 2000). A better understanding of conserved structures and post-transcriptional or post-translational mechanisms that cause an immune response will be useful to determine why some antigens are allergenic and others are not. For instance, questions on how cysteine residues and disulphide bridges are associated with allergenicity or protein function are interesting to address. As many disulphide bridges are strongly conserved through different grass allergens despite lack of a clear common ancestral gene, it is unclear how they pertain to a specific functional or structural role, or whether they were conserved due to a lack of selective pressure.

Research on the specific expression pattern and protein localisation

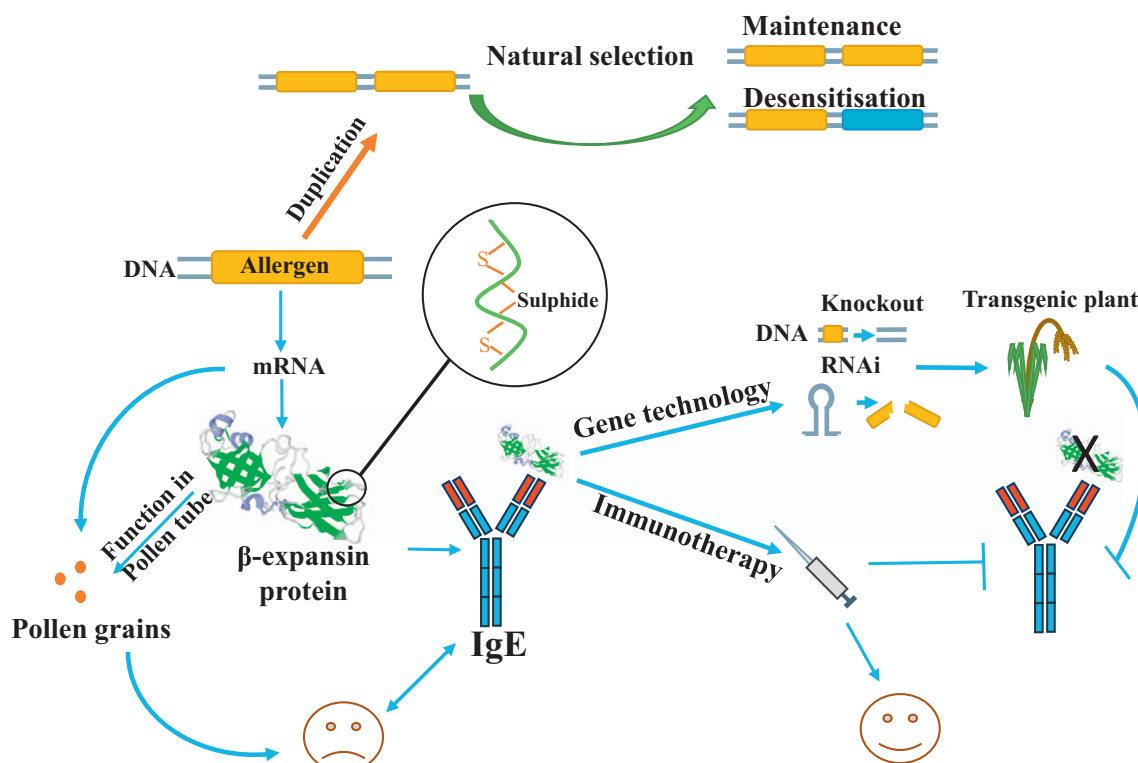


Fig. 5. Summary of Allergen biology and therapy. An Allergen gene (yellow) can evolve in multiple ways. The DNA is transcribed into mRNA and translated into a β -expansin protein, which has conserved cysteine bridges. The allergen may be expressed in pollen and cause an allergenic reaction in patients, confirmed through IgE binding tests. The allergen can be modified using gene technology methods to develop a transgenic plant, or modified for allergen immunotherapy use. The allergen derivative must then be tested for IgE and immunological tests to ensure no reaction occurs in allergy patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of allergen proteins will also be critical for allergen prevention. Lol p 1 and Lol p 5 in ryegrass localise to the intine wall, so localisation data could reveal whether localisation of allergens or proximity to the pollen grain surface plays a role in access of IgE to protein (Suphioglu et al., 1992). Furthermore, expression data may help answer whether some allergens vary in potency due to protein concentration or gene sequence, or whether IgE preferentially binds to proteins located in a particular tissue. This knowledge is essential to the efficient isolation of native allergens for immunotherapy.

The capacity for rational design of AIT could be further improved by understanding the relationship between different allergen structures and patient sensitivity. It was reported that adults with wheat grain allergies were more likely to be sensitized to wheat pollen, suggesting that some allergens within grain are also expressed in pollen or that grain and pollen allergens may share common features (Armentia et al., 2002). Potentially, these shared features could be used to identify and to isolate known food allergens and modifying these shared features them for pollen and grain allergen AIT or gene silencing.

Hypoallergenic plants may provide relief for patients suffering from pollen allergies, but it is necessary for these transgenic plants to retain normal biological function and support plant development in a manner comparable to wild type strains. Alternative and simpler methods such as CRISPR-Cas9 technology may aid in developing gene-site-specific mutations that allow for pollen growth while decreasing allergen-IgE reactivity in patients. The CRISPR-Cas9 system may provide exciting pathways into altering allergenicity of proteins in grasses while retaining normal function and development of the plant.

12. Summary

Biotechnological and immunotherapy approaches against prevalent grass induced allergic diseases have been extensively researched, but

little is currently known about the evolution and function of allergens and this hinders the development of faster, safer and more targeted AIT and biologically viable transgenic hypoallergenic plants (Fig. 5). In this review, we summarized the prevalence and variation of Poaceae species and genes in the context of allergenicity, and pointed out the lack of knowledge on the evolution and function of genes encoding putative allergens in the Poaceae family. Beyond current clinical use of AIT for temperate grass pollen allergy, including new treatments based on whole allergen extracts, AIT for other common Poaceae allergens requires further research, as many species do not yet have an efficient AIT therapy. Finally, genetically modified hypoallergenic plants are promising but have not yet been developed to completely silence allergen genes in pollen and grains. Complete knowledge about allergen function and immune responses will be key to the development of new methods for AIT and transgenic hypoallergenic crops. Exciting new technologies such as genome editing may also provide a pathway for developing new hypoallergenic grasses for the reduction and prevention of allergenicity.

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