Method for producing high-quality recombinant allergens in a plant

The present invention relates to a method for producing high-quality recombinant allergens.

The use of recombinant allergens allows greater specificity and better efficacy of diagnostic tests and treatment of allergies.

Numerous allergens have already been produced in recombinant form. Today they are used for in vitro diagnosis of allergies. However, the expression system used, generally *E. coli*, most often only allows very approximate copies of the natural allergens to be obtained, owing to the inability of this bacterium to perform the post-translational modifications necessary for correct folding of proteins of eukaryotes. This often has an adverse effect on the reliability and sensitivity of the diagnostic tests carried out using these molecules, since certain epitopes able to react with patients' immunoglobulins E (IgE) are not present on the recombinant allergens produced in *E. coli*.

Eukaryotic expression systems have also been used for producing recombinant allergens. These are most often yeasts, and in this case hyperglycosylation, specific to these organisms, still does not allow production of recombinant allergens conforming with their natural homologs.

Plants are the only eukaryotic hosts allowing production of complex allergens in recombinant form, with production costs and quality compatible with their use for personalized treatment of allergies, integrating diagnostic tests and therapy.

However, the plant expression systems used so far for producing recombinant allergens generally employ plant transgenesis with its fundamental limits that are well known, namely:

- long times for transition from the gene to the protein, meaning that the development work takes several years, and
- low yields, of the order of 0.1% to 1% of the soluble proteins, meaning processing of a large volume of biomass of plant material for large-scale production.

Recent advances achieved using transient expression have made it possible to exceed these limits, on the one hand by greatly reducing the delays in passage from the gene to the protein, which allows much faster development, and on the other hand by increasing the production

yields by at least a factor of 10, which minimizes the costs for extraction and purification of the protein of interest.

A technique of this kind for transient expression in plants is now used on a large scale for producing vaccines by certain companies, which are currently developing extensive production units in the United States.

However, despite these efforts, there is still a need for an efficient and reproducible method for producing recombinant allergens that allows recombinant allergens to be obtained having a composition and conformation similar to those of their natural homologs. Moreover, there is a need for a method that has a good production yield.

SUMMARY

In one aspect, the present invention refers to a method for producing a recombinant allergen in a tobacco plant, preferably Nicotiana benthamiana, comprising the following steps: a) culture of the plant aeroponically or hydroponically, and under LED lighting, then b) agroinfiltration of the plant obtained in a), under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant allergen, said DNA fragment being incorporated in an expression vector comprising the SEQ ID NO: 21, then c) returning the plants to culturing after step b), in the same conditions as for step a), then d) extraction and purification of the recombinant allergen from the aerial parts of the plants produced in step c).

The present invention makes it possible to obtain complex recombinant proteins, in particular complex recombinant allergens, which have never been obtained previously in recombinant form. These allergens are, moreover, copies identical to their natural homologs.

The present invention therefore relates to a method for producing a recombinant protein in a plant, preferably a tobacco plant, preferably *Nicotiana benthamiana*, comprising the following steps:
a) culturing the plants aeroponically or hydroponically, preferably on mobile floats, and under LED lighting,
b) agroinfiltration of the plants obtained in step a), under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant protein, then
c) returning the plants to culturing after step b), in the same conditions as for step a), then
d) extraction and purification of the recombinant protein from the aerial parts of the plants produced in step c).

The present invention also relates to a method for producing a recombinant allergen in a plant, notably a tobacco plant, preferably *Nicotiana benthamiana*, comprising the following steps:
a) culture of the plant aeroponically or hydroponically, and under LED lighting, then
b) agroinfiltration of the plant obtained in a), under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant allergen, then
c) returning the plants to culturing after step b), in the same conditions as for step a), then
d) extraction and purification of the recombinant allergen from the aerial parts of the plants produced in step c).

The present invention also relates to a recombinant protein obtainable by the method according to the invention.

The present invention also relates to an expression vector comprising the SEQ ID NO: 21 and an insert, said insert comprising at least one nucleic acid sequence coding for a peptide selected from SEQ ID NO: 1 to 20, said nucleic acid sequence being directly fused with a second nucleic acid sequence encoding the protein of interest.

The present invention also relates to a method for producing a recombinant allergen in a plant, notably a tobacco plant, preferably *Nicotiana benthamiana*, comprising the following steps:
a) culture of the plant aeroponically or hydroponically, preferably on mobile floats, and under LED lighting, then
b) agroinfiltration of the plant obtained in a), under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant allergen, then

c) returning the plants to culturing after step b), in the same conditions as for step a), then

d) extraction and purification of the recombinant allergen from the aerial parts of the plants produced in step c).

The plant usable in the method according to the invention is notably a tobacco plant selected from *Nicotiana benthamiana* and *Nicotiana tabacum* or any other plant usable for transient expression, such as a lettuce (genus Lactuca) or a spinach plant (Spinacia oleracea). Among the lettuces, we may mention the lettuce Appia, Grosse Blonde Paresseuse, Lollo Rosso, Merveille

de quatre saisons ["four-seasons Wonder"], feuille de chêne [oak leaf lettuce], or red sails. The plant may also be of the genus Arabidopsis, or a mutant thereof, in particular glycosylation mutants of Arabidopsis; finally, it is possible to use knock-out tobacco plants (especially of glycosylation mutants).

Preferably, the recombinant protein produced by the method according to the invention is a recombinant allergen, preferably a recombinant mite allergen.

"Allergen" means any protein or any peptide capable of triggering an allergic reaction in a subject previously sensitized when he is in contact with it, most often by contact with the skin, inhalation or ingestion. An allergen is said to be "major" when a purified antigen triggers an allergy in 50% or more of the patients tested, and when it displays specific IgEs, with immediately positive skin tests, at a very low concentration, in at least 70% of subjects having an allergy to this allergen.

"Protein" means a sequence comprising at least 50 amino acids.

"Peptide" means a sequence comprising between 1 and 49 amino acids, preferably between 2 and 40 amino acids.

Preferably, the recombinant protein produced by the method according to the invention is an allergen, an allergen fragment or a fusion protein comprising an allergen or an allergen fragment.

Preferably, the recombinant protein is selected from the allergens responsible for respiratory allergies due to house dust mites, such as *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* or *Euroglyphus manei*, the allergens of storage mites such as *Blomia tropicalis*, the allergens of mites of the type *Acarus siro* (formerly called *Tyroglyphus farinae*), cockroach allergens, tree or grass pollen allergens, allergens from animals (cat, dog, horse), allergens of molds, the allergens responsible for contact allergies such as those of hevea latex or the allergens responsible for food allergies (milk, egg, fish, fruit).

Among the allergens of *Dermatophagoides farinae*, we may mention Der f 10, Der f 11, Der f 13, Der f 14, Der f 15, Der f 16, Der f 17, Der f 18, Der f 2, Der f 2.0101, Der f 2.0102, Der f 2.0103, Der f 2.0104, Der f 2.0105, Der f 2.0106, Der f 2.0107, Der f 2.0108, Der f 2.0109, Der f 2.0110, Der f 2.0111, Der f 2.0112, Der f 2.0113, Der f 2.0114, Der f 2.0115, Der f 2.0116, Der f 2.0117, Der f 20, Der f 3, Der f 4, Der f 5, Der f 6, Der f 7, Der f 8, Der f 9 and Der f HSP70.

Among the allergens of *Dermatophagoides pteronyssinus*, we may mention Der p 10, Der p 11, Der p 14, Der p 15, Der p 18, Der p 2, Der p 2.0101, Der p 2.0102, Der p 2.0103, Der p 2.0104, Der p 2.0105, Der p 2.0106, Der p 2.0107, Der p 2.0108, Der p 2.0109, Der p 2.0110, Der p 2.0111, Der p 2.0112, Der p 2.0113, Der p 20, Der p 21, Der p 3, Der p 4, Der p 5, Der p 6, Der p 7, Der p 8, Der p 9.

Among the allergens of *Blomia tropicalis*, we may mention Blo t 1, Blo t 5 (which has 40% sequence homology with Der p 5), Blo t 9, Blo t 10, Blo t 12 or Blo t 21.

All these allergens are well known, and their sequence may be found notably in databases such as Allergome (allergome.org), or quite simply in UniProt.

The method for producing recombinant allergens by transient expression according to the invention, notably in *N. benthamiana*, is very effective, reproducible, and has a good yield.

The method for producing recombinant proteins according to the invention comprises a first step of culturing the plant (step a), aeroponically or hydroponically, preferably in culture on free mobile floats, and under LED lighting, in order to cause it to produce the recombinant protein.
Aeroponics corresponds to culture of the plant on a support, generally made of plastic, combined with constant misting with nutrient solutions based on mineral salts.
Hydroponics corresponds to culture of the plant without soil. The plant is cultured on a neutral, inert substrate, such as sand, clay beads, polystyrene plates or rock wool. The substrate is regularly irrigated with a stream of solution that supplies mineral salts and essential nutrients to the plant. In the method used according to the invention, tobacco plants, notably *N. benthamiana*, are cultured preferably hydroponically on free floats, for example on a plate of perforated polystyrene. These floats are arranged in tanks containing a culture medium that is constantly aerated by air diffusers. This technique allows standardization of the conditions for production of the recombinant proteins, combined with complete absence of risk of contamination of the agroinfiltration media in step b) by impurities or debris from the substrates contained in the pots (in the case of conventional culture). Moreover, the use of this culture system makes it possible to reach far higher yields, as is shown in the examples.

Finally, during scale-up, manipulation for agroinfiltration or harvesting of batches of plants fixed on a polystyrene plate is of course easier than that of pot and substrate cultured plants.

The method for producing recombinant proteins according to the invention comprises, after step a), a step b) of agroinfiltration of the plant, notably of the tobacco plant, under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant protein.

Notably, after five weeks of culture, preferably hydroponically on free mobile floats, agroinfiltration of the tobacco plants is carried out under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant protein.

This step b) of agroinfiltration may be carried out by any means allowing creation of a vacuum. Preferably, in the method used according to the invention, it is carried out under vacuum by the Venturi effect.

The DNA fragment coding for the recombinant protein, used in step a) and inserted in the agrobacteria, may be prepared by cloning. This DNA fragment may comprise the sequence coding for the recombinant protein, for example a heterologous allergen, said sequence being fused with a sequence encoding a peptide facilitating its purification, for example a "histidine tag" sequence, or a sequence encoding a peptide or a polypeptide for intracellular addressing. Such a peptide or polypeptide for intracellular addressing may notably be selected from the peptides whose sequences are presented in Table 1, i.e. from the peptides SEQ ID NO: 1 to 20. The DNA fragment may then be integrated in the pAG01 expression vector developed in the context of the invention (Fig. 1 and sequence SEQ ID NO: 21), and then the agrobacteria are transformed using this expression vector. Preferably, the invention also relates to an expression vector comprising the sequence SEQ ID NO: 21 and an insert, notably located between the right and left bounds of the transfer DNA (TDNA) (this is illustrated in Fig. 1, where the left bound is "LB", and the right bound is "RB"), said insert comprising at least one nucleic acid sequence coding for a peptide selected from SEQ ID NO: 1 to 20, said nucleic acid sequence being directly fused with a second nucleic acid sequence encoding the protein of interest. This vector corresponds to the pAG01 vector comprising an insert, said insert comprising a nucleic acid peptide sequence (selected from SEQ ID NO: 1 to 20) directly fused with the nucleic acid sequence of the allergen of interest. Preferably, the protein of interest is an allergen as described above.

Agroinfiltration of the aerial parts of plants, notably of tobacco plants, notably of *N. benthamiana*, is carried out under vacuum. Preferably, an air-tight chamber is used, which has a system for vacuuming by the Venturi effect. Typically, the chamber contains the culture of agrobacteria and after inverting the floating platforms on which the plants are cultured hydroponically, the latter are immersed, upside down, in the bacterial suspension. This method is illustrated in Fig. 2. It allows simultaneous infiltration of all the plants cultured on one and the same floating platform.

According to a first embodiment, agroinfiltration is carried out by a step of putting the plants under vacuum for 2 minutes.

Preferably, according to a second embodiment, agroinfiltration is carried out in three steps (sequential process):

1) putting under vacuum, preferably at -0.8 bar for 2 minutes,

2) breaking the vacuum, and return to atmospheric pressure preferably for 30 seconds, then

3) putting under vacuum, preferably at -0.8 bar for 2 minutes, followed by return to atmospheric pressure.

This agroinfiltration technique is quick (total duration less than 5 minutes), effective, and easy to automate.

Among the agrobacteria usable according to the invention, we may mention preferably the strains LBA4404, GV3101, EHA 101/105 or C58.

Preferably, the agrobacteria are used for infiltration at a concentration defined by an OD600 between 0.7 and 1.0, in a solution comprising 10 mM of Mes (2-morpholino-ethanesulfonic acid), which may optionally be substituted with MOPS (3-(N-morpholinopropanesulfonic acid), 10 mM of $MgCl_2$ and 100µM of acetosyringone.

At the end of step b) of agroinfiltration, the method comprises a step c) of returning the plants to culturing, in the same conditions as for step a).

The plants are typically drained upside down for 15 minutes, then put back in culture in the conditions described for step a), ideally ensuring frequent misting of the latter for the first 6 hours of culture following agroinfiltation. Alternatively, the plants are put directly back in culture in the conditions described for step a).

Finally, the method according to the invention comprises a step d) of extraction and purification of the recombinant protein produced after agroinfiltration in step c).

The plant biomass is harvested 4 to 5 days after putting the plants in culture following agroinfiltration.

After grinding and extraction of the proteins from the aerial parts of the plants, the recombinant protein is purified. The techniques of extraction and purification known from the prior art may be employed in this step. Preferably, if the recombinant protein comprises a "histidine tag" sequence, it is purified by immobilized nickel column chromatography (IMAC), followed by a step of molecular sieving. The tag sequence used for purification may then be cleaved from the end product.

The invention will now be illustrated with the following examples, which are not limiting.

The legends of the figures are as follows:

Table 1 (Fig. 8): Reozyme™ sequences used for targeted expression of recombinant allergens and subcellular storage compartment of the allergens when they are produced in fusion with these addressing peptides. ER: endoplasmic reticulum; GA: Golgi apparatus.

Fig. 1: The T-DNA of the pAG01 vector consists of the two flanking sequences of the T-DNA of the agrobacteria (RB and LB), and of three expression cassettes allowing silencing inhibitor expression (cassette 1), of a recombinant protein preferably an allergen (cassette 2) and of an enzyme conferring resistance to a selection antibiotic or of a protein maturation enzyme (cassette 3).

Fig. 2: The platform developed according to the invention combines original steps and tools that allow low-cost mass production of recombinant allergens of unequalled high quality. Adaptability and speed of production, since 4 to 5 days is sufficient to go from the gene to the protein, are also features of this production platform.

Fig. 3: Production of complex major allergens of the mite *Dermatophagoides pteronyssinus*: Der p 4 (track 1); Der p 7 (track 2); Der p 21 (track 4); Der p 5 (track 6) and Der p 2 (track 7); one of the major allergens of latex: Hev b 13 (track 3) and a mold allergen: CP120 (track 5).

Fig. 4: Schematic illustration of the expression cassettes used (panel A). In panel B, Western blot analyses illustrate the differences in quality of the allergen Der p 2 produced by fusion with different Reozyme™ signals. The allergen is produced in a heterogeneous form and has a noncompliant molecular weight when the Reozyme™ signals R1, R2 and R3 are used. However, when the signal R4 is used, the recombinant allergen is homogeneous and has a molecular weight identical to that of the natural allergen.

Fig. 5: Panel A presents the SDS-PAGE analysis of the total proteins extracted from each plant. Analysis of these extracts by Western blot (panels B and C) illustrates the production yields for one and the same protein of interest, when the plants are cultured either in standard conditions (pot culture + lighting by incandescent lamp) (tracks 1-6, the different tracks correspond to different transformation events), or cultured aeroponically + LED lighting (tracks 7-9). Panel B: chromogenic detection; panel C: detection by chemiluminescence.

Fig. 6: Comparison of expression of the GFP: 1) when agroinfiltration is carried out according to the protocol described in the invention (panel B), or 2) when infiltration is carried out according to a conventional method of infiltration (panel A).

Fig. 7: Analysis by SDS-PAGE and Western Blot of the steps of purification of the allergen Der p 4.

Track 1: Total protein extract.

Track 2: Der p 4 purified on the immobilized nickel column (IMAC) and eluted in the presence of 50 mM of imidazole

Track 3: Der p 4 purified by molecular sieving after the IMAC step.

Track 4: Der p 4 purified, whose tag was cleaved in vitro.

Top panel: Analysis of Der p 4 by SDS-PAGE followed by staining of the proteins with Coomassie Blue in the gel.

Bottom panel: Analysis of Der p 4 by SDS-PAGE followed by Western blotting and immunodetection on the print with a specific immunoserum of the purification tag.

Example 1: Standardized production of complex antigens

For validation of the method described here, cDNAs coding for complex allergens of mites, tree or molds were cloned into the pAG01 vector. These vectors were then inserted in the agrobacteria (strain LBA4404) with a view to transient expression in *N. benthamiana.*

The *N. benthamiana* plants were cultured by the method described below: the seeds are sown in earth and cultured for at most 45 days on this substrate. Preferably, the seedlings from these seeds develop for 15 days in this substrate (under LED lighting), before being transferred to tanks for hydroponic culture on free floats. The plants are then cultured for 25 days in these conditions in the presence of nutrients and trace elements, under LED lighting. In an alternative to this protocol, the use of coated seeds allows direct sowing of the *N. benthamiana* plants on the floating platforms. In these conditions, germination and culture of the plants take place in hydroponic conditions.

After culture for 40 days, the plants maintained on the floats are transferred to an air-tight chamber for transfection. For penetration of the agrobacteria bearing the binary vector, the aerial part of the plants is immersed (float inverted) in the solution of agrobacteria whose concentration corresponds to OD 600: 0.7. Transfection is carried out under vacuum in the air-tight chamber by the Venturi effect according to the following protocol: 2 min under vacuum (-0.8 bar), return to normal, then again 2 min under vacuum (-0.8 bar). The floats are then put on supports (plants upside down) for 10 to 15 min, so that the plants drain. Then the plants, still held on the floating platforms on which they were cultured, are put back in the culture tanks for 4 days, ideally ensuring frequent misting of the latter for the first 6 hours of culture following agroinfiltation.

After these 4 days, the aerial parts of the plants expressing the various allergens are harvested. The proteins are extracted in a denaturing buffer and then analyzed by SDS-PAGE and/or Western blotting using an antibody directed against the FLAG epitope.

Fig. 3 illustrates the results obtained. The method according to the invention allows production of complex major allergens.

Example 2: The method according to the invention allows quality control of the allergens

In order to monitor the maturation and the homogeneity of the recombinant allergens, different signals (R1, R2, R3 and R4) were fused with the allergen of interest, and then the fusion protein was cloned into the pAG01 vector. These vectors were then inserted in the agrobacteria (strain LBA4404) with a view to transient expression in *N. benthamiana*.

The *N. benthamiana* plants were cultured by the method described below: the seeds are sown in earth and the seedlings from these seeds develop for 15 days in this substrate (LED lighting) before being transferred to free floats. The plants are then cultured for 25 days hydroponically on free floats.

After culture for 40 days in these conditions, the plants maintained on the floating culture platforms are transferred to an air-tight chamber for transfection. For penetration of the agrobacteria bearing the binary vector, the aerial parts of the plants are immersed (float inverted) in the solution of agrobacteria. Transfection is carried out under vacuum in the air-tight chamber by the Venturi effect according to the following protocol: 2 min under vacuum (-0.8 bar), return to normal, then again 2 min under vacuum (-0.8 bar). The floats are then put on supports (plants upside down) for 10 to 15 min so that the plants drain. Then the plants, still held on the floats, are put back in the culture tanks for 4 days.

After these 4 days, the plants expressing the various allergens are harvested. The proteins are extracted by grinding the aerial parts in a denaturing buffer and then analyzed by SDS-PAGE and/or Western blotting using an antibody directed against the FLAG epitope.

Fig. 4 presents a schematic illustration of the expression cassettes used (panel A). It also shows, with the example of the mite allergen Der p 2, the qualitative advantages associated with using the Reozyme™ signals. In fact, in panel B, Western blot =analyses illustrate the differences in quality of the allergen Der p 2 produced by fusing with different Reozyme™ signals. The allergen is produced in a heterogeneous form and has a noncompliant molecular weight when the Reozyme™ signals R1, R2 and R3 are used. However, when signal R4 is used, the recombinant allergen is homogeneous and has a molecular weight identical to that of the natural allergen.

Example 3: The method according to the invention allows a higher yield

For this example, we compared the use of the pAG01 vector coupled with the use of the method described in the invention with the use of a binary vector (-/+ silencing inhibitor) coupled to conventional methods of transfection described for example in Medrano et al. (2009).

The cDNA encoding the allergen Der p 7 of *Dermatophagoides pteronyssinus* was cloned either into the pAG01 vector, or into the pBI121 vector. These vectors were then inserted in the agrobacteria (strain LBA4404) with a view to transient expression in *N. benthamiana.*

Then the strains of agrobacteria were used for transfecting plants cultured either hydroponically on floating platforms as described in examples 1 and 2, or cultured in earth and then infiltrated under vacuum according to a conventional protocol as published in Pogue et al. (2010).

As illustrated in Fig. 5, the plant culture conditions described in the method, as well as the use of the pAG01 vector, allow higher yields of recombinant allergens than those observed with the conditions generally used for transient expression.

It can clearly be seen from Fig. 5 that the yields of the method according to the invention are far higher than those obtained with a conventional method. Moreover, the inventors demonstrate better homogeneity of the various transformation events, as illustrated by tracks 7 to 9 compared to tracks 4 to 6.

The higher expression levels observed for Der p 7 are partly explained by the conditions of agroinfiltration according to the method of the invention. In fact, as illustrated in Fig. 6, infiltration of the foliar tissues is more effective, as is described in the method. In this figure, the inventors compared the expression of GFP: 1) when agroinfiltration is carried out according to the protocol described in the invention (panel B), or 2) when infiltration is carried out according to a conventional method of infiltration (panel A) described for example in Medrano et al. (2009).

Example 4: The method according to the invention allows easy purification

The leaves of the *Nicotiana benthamiana* plants are collected, and the proteins are extracted by grinding this plant material in a phosphate buffer supplemented with NaCl (0.1 M), pH 7.5. After quick filtration, the extract is deposited on an immobilized nickel column. The proteins of the extract that do not have affinity for the chromatography matrix are not retained on the column. However, the recombinant allergens produced according to the method have a hexa-histidine tag and are retained on this type of matrix. After washing the column to remove the contaminating proteins, the allergens are eluted specifically in the presence of 50 mM imidazole in phosphate buffer.

The method of production according to the invention is flexible and easily adaptable to the production of any allergen of interest. This is true not only of the techniques for culture, cloning, agroinfiltration, and extraction, but also for purification.

In fact, owing to fusion of a tag, purification of the recombinant allergens is standardized. This is illustrated in Fig. 7, which presents analysis by SDS-PAGE and Western blotting of the steps of purification of the allergen Der p 4 produced as described in the invention. This analysis illustrates the method of purification of this allergen in two chromatography steps: 1) immobilized nickel affinity column (IMAC) and 2) molecular sieving.

Track 1: Total protein extract.

Track 2: Der p 4 purified on the immobilized nickel column (IMAC) and eluted in the presence of 50 mM of imidazole

Track 3: Der p 4 purified by molecular sieving after the IMAC step.

Track 4: Der p 4 purified, whose tag was cleaved in vitro.

Top panel: Analysis of Der p 4 by SDS-PAGE followed by staining of the proteins with Coomassie Blue in the gel.

Bottom panel: Analysis of Der p 4 by SDS-PAGE followed by Western blotting and immunodetection on the print with a specific immunoserum of the purification tag.

CLAIMS

1. A method for producing a recombinant allergen in a tobacco plant, preferably *Nicotiana benthamiana*, comprising the following steps:
a) culture of the plant aeroponically or hydroponically, and under LED lighting, then
b) agroinfiltration of the plant obtained in a), under vacuum, by agrobacteria comprising a DNA fragment coding for the recombinant allergen, said DNA fragment being incorporated in an expression vector comprising the SEQ ID NO: 21, then
c) returning the plants to culturing after step b), in the same conditions as for step a), then
d) extraction and purification of the recombinant allergen from the aerial parts of the plants produced in step c).

2. The method as claimed in claim 1, characterized in that the recombinant allergen is a recombinant mite allergen.

3. The method as claimed in claim 1 or 2, characterized in that the recombinant allergen is selected from the allergens of *Dermatophagoides farinae*, the allergens of *Dermatophagoides pteronyssinus*, the allergens of *Euroglyphus manei*, the allergens of *Acarus siro*, the allergens of *Blomia tropicalis*, cockroach allergens, tree or grass pollen allergens, allergens of animals, allergens of molds, allergens of hevea latex and allergens responsible for food allergies.

4. The method as claimed in any one of claims 1 to 3, characterized in that the recombinant allergen is selected from Der f 10, Der f 11, Der f 13, Der f 14, Der f 15, Der f 16, Der f 17, Der f 18, Der f 2, Der f 2.0101, Der f 2.0102, Der f 2.0103, Der f 2.0104, Der f 2.0105, Der f 2.0106, Der f 2.0107, Der f 2.0108, Der f 2.0109, Der f 2.0110, Der f 2.0111, Der f 2.0112, Der f 2.0113, Der f 2.0114, Der f 2.0115, Der f 2.0116, Der f 2.0117, Der f 20, Der f 3, Der f 4, Der f 5, Der f 6, Der f 7, Der f 8, Der f 9 and Der f HSP70.

5. The method as claimed in any one of claims 1 to 3, characterized in that the recombinant allergen is selected from Der p 10, Der p 11, Der p 14, Der p 15, Der p 18, Der p 2, Der p 2.0101, Der p 2.0102, Der p 2.0103, Der p 2.0104, Der p 2.0105, Der p 2.0106, Der p 2.0107,

Der p 2.0108, Der p 2.0109, Der p 2.0110, Der p 2.0111, Der p 2.0112, Der p 2.0113, Der p 20, Der p 21, Der p 3, Der p 4, Der p 5, Der p 6, Der p 7, Der p 8 and Der p 9.

6. The method as claimed in any one of claims 1 to 3, characterized in that the recombinant allergen is selected from Blo t 1, Blo t 5, Blo t 9, Blo t 10, Blo t 12 and Blo t 21.

7. The method as claimed in any one of claims 1 to 6, characterized in that the agroinfiltration is carried out under vacuum by the Venturi effect.

8. The method as claimed in claim 7, characterized in that the agroinfiltration is carried out either by a step of putting the plant under vacuum for 2 minutes, or by vacuuming, then breaking the vacuum and returning to atmospheric pressure, then putting under vacuum, followed finally by return to atmospheric pressure.

9. The method as claimed in any one of claims 1 to 8, characterized in that the expression vector further comprises an insert, said insert comprising at least one nucleic acid sequence coding for a peptide selected from SEQ ID NO: 1 to 20, said nucleic acid sequence being directly fused with the DNA fragment coding for the recombinant allergen.

10. The method as claimed in claim 9, characterized in that the allergen is selected from the allergens of *Dermatophagoides farinae*, the allergens of *Dermatophagoides pteronyssinus*, the allergens of *Blomia tropicalis*, the allergens of *Euroglyphus manei*, the allergens of *Acarus siro*, cockroach allergens, tree or grass pollen allergens, allergens of animals, allergens of molds, allergens of hevea latex and allergens responsible for food allergies.