Expression of fungicide-binding polypeptides in plants for generating fungicide tolerance

The present invention relates to a process for the production of fungicide-tolerant plants by expressing an exogenous fungicide-binding polypeptide in plants or plant organs. The invention furthermore relates to the use of the corresponding nucleic acids which encode a polypeptide, an antibody or parts of an antibody with fungicide-binding properties in transgenic plants, and the thus transformed plant itself.

It is known that genetic engineering methods allow the specific transfer of foreign genes into the genome of a plant. This process is termed transformation, and the resulting plants transgenic plants. Transgenic plants are currently being employed in various fields of biotechnology. Examples of insect-resistant plants (Vaek et al. Plant Cell 5 (1987), 159-169), virus-resistant plants (Powell et al. Science 232 (1986), 738-743) and ozone-resistant plants (Van Camp et al. BioTech. 12 (1994), 165-168). Examples of improved quality characteristics achieved by genetic engineering are: improved shelf life of fruit (Oeller et al. Science 254 (1991), 437-439), increased starch production in potato tubers (Stark et al. Science 242 (1992), 419), changes in starch (Visser et al. Mol. Gen. Genet. 225 (1991), 289-296) and lipid composition (Voelker et al. Science 257 (1992), 72-74), and production of foreign polymers (Poirer et al. Science 256 (1992), 520-523).

An important target of work carried out in the field of plant molecular genetics is the generation of herbicide tolerance. Herbicide tolerance is characterized by an improved compatibility (in terms of type or level) of the plant or plant organs with the herbicide applied. This can be effected in various ways. The known methods are utilization of a metabolic gene, for example the pat gene, in connection with glufosinate resistance (WO 8705629) or a target enzyme which is resistant to the herbicide, such as in the case of enolpyruvyl shikimate-3-phosphate synthase (WO 9204449), which is resistant to glyphosate, and the use of a herbicide in cell and tissue culture for the selection of tolerant plant cells and resulting resistant plants, such as described in the case of acetyl-CoA-carboxylase inhibitors (US 5162602, US 5290696).

Antibodies are proteins as component of the immune system. A joint feature of all antibodies is their spatial, globular

structure, the construction of light and heavy chain and their basic capability of binding molecules or parts of a molecular structure with high specificity (Alberts et al., in: Molekularbiologie der Zelle [Molecular Biology of the Cell], 2nd Edition 1990, VCH Verlag, ISBN 3-527-27983-0, 1198-1237). On the basis of these properties, antibodies have been utilized for a number of tasks. Application can be divided into application of the antibodies within the animal and human organisms in which they are produced, that is to say the so-called in-situ applications, and the ex-situ applications, ie. utilization of the antibodies after they have been isolated from the producing cells or organisms (Whitelam und Cockburn, TIPS Vol.1 , 8 (1996), 268-272).

The use of somatic hybrid cell lines (hybridomas) as a source of antibodies against very specific antigens is based on work carried out by Köhler and Milstein (Nature 256 (1975) 495-97). This process allows so-called monoclonal antibodies to be produced which have a uniform structure and which are produced by means of cell fusion. Spleen cells of an immunized mouse are fused with mouse myeloma cells. This gives hybridoma cells which multiply infinitely. At the same time, the cells secrete specific antibodies against the antigen with which the mouse had been immunized. The spleen cells provide the capability of antibody production while the myeloma cells contribute the capacity of unlimited growth and continuous secretion of antibodies. Since each hybridoma cell, being a clone, is derived from a single B cell, all antibody molecules produced have the same structure, including the antigen binding site. This method has greatly promoted the use of antibodies since antibodies which have a single, known specificity and a homogeneous structure are now available in unlimited quantities. Monoclonal antibodies are used widely in immunodiagnostics and as therapeutics.

In recent years, the so-called phage display method has become available for the production of antibodies, and here the immune system and the various immunizations in the animal are avoided. The affinity and specificity of the antibody are made to measure in vitro (Winter et al., Ann. Rev. Immunol. 12 (1994), 433-455; Hoogenboom TIBTech Vol 15 (1997), 62 -70). Gene segments which contain the sequence which encodes the variable region of antibodies, ie. the antigen binding site, are fused with genes for the coat protein of a bacteriophage. Then, bacteria are infected with phages which contain such fusion genes. The resulting phage particles are now equipped with coats containing the antibody-like fusion protein, the antibody-binding domain pointing outward. Such a phage display library can now be used

for isolating the phage which contains the desired antibody fragment and which binds specifically to a certain antigen. Each phage isolated in this manner produces a monoclonal antigen-binding polypeptide which corresponds to a monoclonal antibody. The genes for the antigen binding site, which are unique for each phage, can be isolated from the phage DNA and employed for constructing complete antibody genes.

In the field of crop protection, antibodies were utilized in particular as analytical tools ex-situ for the qualitative and quantitative detection of antigens. This includes the detection of plant constituents, herbicides or fungicides in drinking water (Sharp et al. (1991) ACS Symp Ser., 446 (Pestic. Residues Food Saf.) 87-95), soil samples (WO 9423018) or in plants or plant organs, and the utilization of antibodies as auxiliaries for the purification of bound molecules.

The production of immunoglobulins in plants was first described by Hiatt et al., Nature, 342 (1989), 76 - 78. The spectrum encompasses single-chain antibodies up to multimeric secretory antibodies (J. Ma and Mich Hein, 1996, Annuals New York Academy of Sciences, 72 - 81).

More recent attempts utilize antibodies in-situ for defending plants against pathogens, in particular viral diseases, by expressing, in plant cells, specific antibodies or parts thereof which are directed against viral coat proteins (Tavladoraki et al., Nature 366 (1993), 469-472; Voss et al., Mol. Breeding 1 (1995), 39-50).

An analogous approach has also been utilized for defending the plant against infection by nematodes (Rosso et al., Biochem Biophys Res Com, 220 (1996) 255-263). There exist examples for an application in pharmacology where the in-situ expression of antibodies in plants is utilized for oral immunization (Ma et al., Science 268 (1995), 716-719; Mason and Arntzen, Tibtech Vol 13 (1996), 388-392). The body is provided with antibodies formed by the plant and originating from plants or plant organs which are suitable for consumption, via the mouth, throat or digestive tract, which antibodies cause efficient immunoprotection. Moreover, a single-chain antibody against the low-molecular-weight plant hormone abscisic acid has already been expressed in plants, and a reduced availability of plant hormones, due to binding of abscisic acid in the plant, has been observed (Artsaenko et al., The Plant Journal (1995) 8(5), 745-750).

Chemical control of fungi in agronomically important crops requires the use of highly selective fungicides without phytotoxic effect. The phytotoxic effect of fungicides may be based, for example, on inhibition of plant growth, reduced photosynthesis and thus reduced yield. However, in some cases it is difficult to develop sufficiently selective fungicides which can be employed in all important large-scale crops of plants and which do not cause damage of the plant which provides the yield in any crop. The introduction of fungicide-resistant or -tolerant crop plants can contribute to solving this problem and can open up novel uses for fungicides in crops where treatment has not been possible to date, or has been possible but only when yield losses were acceptable.

The development of fungicide-resistant crop plants by tissue culture or seed mutagenesis and natural selection is limited. On the one hand, the phytotoxic effect must already be detectable at the tissue culture level and, on the other hand, only those plants can be manipulated via tissue culture techniques where entire plants can be regenerated successfully from cell cultures. Moreover, following mutagenesis and selection, crop plants may display undesirable characteristics which have to be reeliminated by, in some cases repeated, back-crossing. Also, the introduction of a resistance by performing crosses would be restricted to plants of the same species.

It is for the abovementioned reasons that the genetic engineering approach of isolating a resistance-encoding gene and transferring it into crop plants in a targeted manner is superior to the traditional plant breeding method.

To date, the development of herbicide-tolerant or herbicide-resistant crop plants, by molecular biology methods, requires a knowledge of the mechanism of action of the herbicide in the plant and also that genes which impart resistance to the herbicide can be found. A large number of herbicides which are presently utilized commercially act by blocking an enzyme of an essential amino acid, lipid or pigment biosynthesis step. Herbicide tolerance can be generated by altering the genes of these enzymes in such a way that the herbicide can no longer be bound and by introducing these altered genes into crop plants. An alternative example is to find analogous enzymes in nature, for example in microorganisms which exhibit a natural resistance to the herbicide. This resistance-imparting gene is isolated from such a microorganism, recloned into suitable vectors and subsequently, after successful transformation, expressed in

herbicide-sensitive crop plants (WO 96/38567).

It was an object of the present invention to develop a novel, generally utilizable genetic engineering method for producing fungicide-tolerant transgenic plants.

We have found that this object is achieved, surprisingly, by a process of expressing, in the plants, an exogenous polypeptide, antibody or parts of an antibody with fungicide-binding properties.

The present invention relates firstly to the production of a fungicide-binding antibody and the cloning of the relevant gene or gene fragment.

The first step is to produce a suitable antibody which binds the fungicide. This can be effected, inter alia, by immunizing a vertebrate, in most cases mouse, rat, dog, horse, donkey or goat, with an antigen. The antigen in this case is a fungicidally active compound which is associated or coupled to a higher-molecular-weight carrier such as bovine serum albumin (BSA), chicken ovalbumin, keyhole limpet hemocyanine (KLH) or other carriers, via a functional group. After antigen has been applied repeatedly, the immune response is monitored with customary methods, and a suitable antiserum is thus isolated. Initially, this approach yields a polyclonal serum which contains antibodies with differing specificities. For the targeted in-situ use, it is necessary to isolate the gene sequence which encodes a single, specific, monoclonal antibody. A variety of routes are available for this purpose. The first approach exploits the fusion of antibody-producing cells and cancer cells to give a hybridoma cell culture which continuously produces antibodies and which finally, by singling the clones obtained, leads to a homogeneous cell line which produces a defined monoclonal antibody.

The cDNA for the antibody, or parts of the antibody, viz. the so-called single chain antibody (scFv), is isolated from such a monoclonal cell line. These cDNA sequences can then be cloned into expression cassettes and used for the functional expression in prokaryotic and eukaryotic organisms, including plants.

Alternatively, it is possible to select antibodies via phage display libraries, and these antibodies bind fungicide molecules and convert them catalytically into a product which has non-fungicidal properties. Methods for the production of

catalytic antibodies are described in Janda et al., Science 275 (1997) 945-948, Chemical selection for catalysis in combinatorial Antibody libraries; Catalytic Antibodies, 1991, Ciba Foundation Symposium 159, Wiley- Interscience Publication. Cloning the gene of this catalytic antibody and expressing it in a plant may, in principle, also lead to a fungicide-resistant plant.

The invention particularly relates to expression cassettes whose encoding sequence encodes a fungicide-binding polypeptide or a functional equivalent thereof, and to the use of these expression cassettes for the production of a fungicide-tolerant plant. The nucleic acid sequence can be, for example, a DNA sequence or a cDNA sequence. Encoding sequences which are suitable for insertion into an expression cassette according to the invention are, for example, those which contain a DNA sequence from a hybridoma cell which encodes a polypeptide with fungicide-binding properties and thus impart resistance to specific fungicides to the host.

Moreover, the expression cassettes according to the invention contain regulatory nucleic acid sequences which govern expression of the encoding sequence in the host cell. In a preferred embodiment, an expression cassette according to the invention comprises upstream, ie. on the 5'-end of the encoding sequence, a promoter and downstream, ie. on the 3'-end, a polyadenylation signal and, if appropriate, other regulatory elements which are linked operatively with the in-between encoding sequence for the polypeptide with fungicide-binding properties and/or transit peptide. Operative linkage is to be understood as meaning the sequential arrangement of promoter, encoding sequence, terminator and, if appropriate, other regulatory elements in such a way that each of the regulatory elements can function as intended when the encoding sequence is expressed. The sequences preferred for operative linkage, but not limited thereto, are targeting sequences for guaranteeing subcellular localization in the apoplast, in the plasma membrane, in the vacuole, in plastids, into the mitochondrium, in the endoplasmatic reticulum (ER), in the nucleus, in liposomes or in other compartments and translation enhancers, such as the 5'-leader sequence from the tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987) 8693-8711).

A suitable promotor of the expression cassette according to the invention is, in principle, any promoter which is capable of governing the expression of foreign genes. Promoters which are preferably used are, in particular, a plant-derived promoter or a

promoter originating from a plant virus. Particularly preferred is the CaMV 35S promotor from the cauliflower mosaic virus (Franck et al., Cell 21(1980) 285-294). This promoter contains various recognition sequences for transcriptional effectors, which, in their totality, lead to permanent and constitutive expression of the gene introduced (Benfey et al., EMBO J. 8 (1989) 2195-2202).

The expression cassette according to the invention may also comprise a chemically inducible promoter by means of which expression of the exogenous polypeptide in the plant can be controlled at a particular point in time. Such promoters, for example the PRP1 promotor (Ward et al.,Plant.Mol.Biol.22(1993), 361-366), a promoter which is inducible by salicylic acid (WO 95/1919443), a promoter which is inducible by benzenesulfonamide (EP 388186), a promoter which is inducible by abscisic acid (EP335528) or a promoter which is inducible by ethanol or cyclohexanone (WO9321334), have been described in the literature and can be used, among others.

Other promoters which are particularly preferred are those which guarantee expression in tissues or plant organs in which the phytotoxic fungicidal activity takes place. Promoters which guarantee leaf-specific expression deserve particular mention. Mention must be made of the potato cytosolic FBPase promoter or the potato ST-LSI promotor (Stockhaus et al., EMBO J. 8 (1989) 2445-245).

The stable expression of single-chain antibodies, which amounted to up to 0.67% of the total soluble seed protein in the seeds of transgenic tobacco plants, was made possible with the aid of a seed-specific promoter (Fiedler and Conrad, Bio/Technology 10(1995), 1090-1094). Since expression may also be possible in seeds which have been sown or which are in the process of germination and may be desired for the purposes of the present invention, such germination- and seed-specific promoters are also regulatory elements which are preferred in accordance with the invention. Thus, the expression cassette according to the invention can therefore contain, for example, a seed-specific promoter (preferably the USP or LEB4 promotor), the LEB4 signal peptide, the gene to be expressed, and an ER retention signal. The construction of the cassette is shown by way of example in the form of a diagram in Figure 1 with reference to a single-chain antibody (scFv gene).

An expression cassette according to the invention is produced by fusing a suitable promoter with a suitable polypeptide DNA and, preferably, a DNA which encodes a chloroplast-specific transit peptide and which is inserted between promoter and polypeptide DNA, and a polyadenylation signal, using customary recombination and cloning techniques as they are described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and also in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

Particularly preferred are sequences which allow targeting into the apoplast, the plastid, the vacuole, the plasma membrane, the mitochondrium, the endoplasmatic reticulum (ER) or, by the absence of suitable operative sequences, residence in the compartment of formation, namely the cytosol (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423). Localization in the ER and the cell wall have proved to be especially beneficial for quantitative protein accumulation in transgenic plants (Schouten et al. , Plant Mol. Biol. 30 (1996), 781-792; Artsaenko et al., Plant J. 8 (1995) 745-750).

The invention also relates to expression cassettes whose encoding sequence encodes a fungicide-binding fusion protein, part of the fusion protein being a transit peptide, which governs translocation of the polypeptide. Especially preferred are chloroplast-specific transit peptides which are cleaved enzymatically from the fungicide-binding polypeptide moiety after the fungicide-binding polypeptide has been translocated into the plant's chloroplasts. Particularly preferred is the transit peptide derived from plastid transketolase (TK) or a functional equivalent of this transit peptide (for example the transit peptide of the small subunit of Rubisco or ferredoxin NADP oxidoreductase).

The polypeptide DNA or polypeptide cDNA required for the production of expression cassettes according to the invention is preferably amplified with the aid of polymerase chain reaction (PCR). DNA amplification methods using PCR are known, for example from Innis et al., PCR Protocols, A Guide to Methods and Applications, Academic Press (1990). The PCR-produced DNA fragments can expediently be checked by sequence analysis to

avoid polymerase errors in constructs to be expressed.

The nucleotide sequence inserted, which encodes a fungicide-binding polypeptide, can be prepared synthetically or obtained naturally or comprise a mixture of synthetic and natural DNA components. In general, synthetic nucleotide sequences with codons which are preferred by plants are prepared. These codons which are preferred by plants can be determined from codons whose proteins are most frequent and which are expressed in most of the interesting plant species. When preparing an expression cassette, various DNA fragments can be manipulated so as to obtain a nucleotide sequence which expediently reads in the correct sense and which is equipped with a correct reading frame. To connect the DNA fragments to each other, adaptors or linkers can be added to the fragments.

The promoter and terminator regions according to the invention should expediently be provided, in the sense of the transcription, with a linker or polylinker comprising one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, usually 1 to 8, preferably 2 to 6, restriction sites. Within the regulatory regions, the linker generally has a size of less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter according to the invention can be either native or homologous or else foreign or heterologous to the host plant. The expression cassette according to the invention comprises, in the 5'-3'-sense of transcription, the promoter according to the invention, any desired sequence and a region for transcriptional termination. Various termination regions are mutually exchangeable as desired.

Furthermore, manipulations which provide suitable restriction sites or which remove excess DNA or restriction sites can be employed. Where insertions, deletions or substitutions, for example transitions and transversions, are possible, in-vitro mutagenesis, "primerre [sic] pair", restriction or ligation may be used. In the case of suitable manipulations such as restriction, "chewing-back" or filling up projections for "blunt ends", complementary ends of the fragments may be provided for ligation purposes.

Especially important for the success according to the invention is the attachment of the specific ER retention signal SEKDEL (Schuoten, A. et al. Plant Mol. Biol. 30 (1996), 781 - 792), with which the average expression level is trebled to quadrupled. Other retention signals which occur naturally in plant and animal

proteins which are localized in the ER may also be used for constructing the cassette.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to T-DNA polyadenylation signals from Agrobacterium tumefaciens, in particular gene 3 of the T-DNA (octopin synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 et seq.) or functional equivalents.

An expression cassette according to the invention may comprise, for example, a constitutive promotor (preferably the CaMV 35 S promotor), the LeB4 signal peptide, the gene to be expressed and the ER retention signal. The construction of the cassette is shown as a diagram in Figure 2 with reference to a single-chain antibody (scFv gene). The amino acid sequence KDEL (lysine, aspartic acid, glutamic acid, leucine) is preferably used as ER retention signal.

The fused expression cassette which encodes a polypeptide with fungicide-binding properties is preferably cloned into a vector, for example pBin19, which is suitable for transforming Agrobacterium tumefaciens. Agrobacteria which are transformed with such a vector can then be used in the known manner for transforming plants, in particular crop plants, eg. tobacco plants, by, for example, bathing wounded leaves or leaf sections in an Agrobacterial solution and subsequently growing them in suitable media. The transformation of plants by means of Agrobacteria is known, inter alia, from F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38, and from S.B. Gelvin, Molecular Genetics of T-DNA Transfer from Agrobacterium to Plants, also in Transgenic Plants, pp. 49-78. Transgenic plants can be regenerated from the transformed cells of the wounded leaves or leaf sections in the known manner, and these transgenic plants contain a gene for the expression of a polypeptide with fungicide-binding properties, integrated into the expression cassette according to the invention.

To transform a host plant with a DNA encoding a fungicide-binding polypeptide, an expression cassette according to the invention is incorporated, as an insertion, into a recombinant vector whose vector DNA contains additional functional regulation signals, for example sequences for replication or integration. Suitable vectors are described, inter alia, in "Methods in Plant Molecular

Biology and Biotechnology" (CRC Press), chapter 6/7, pp.71-119 (1993).

Using the above-cited recombination and cloning techniques, the expression cassettes according to the invention can be cloned into suitable vectors which allow them to be multiplied, for example in E. coli. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Especially suitable are binary vectors which can replicate in both E. coli and agrobacteria, for example pBin19 (Bevan et al. (1980) Nucl. Acids Res. 12, 8711).

The invention furthermore relates to the use of an expression cassette according to the invention for the transformation of plants, plant cells, plant tissues or plant organs. The preferred aim upon use is the mediation of resistance to phytotoxically active fungicides.

Depending on the choice of the promoter, expression can take place specifically in the leaves, in the seeds or in other plant organs. Such transgenic plants, their propagation material and their plant cells, plant tissues or plant organs are a further subject of the present invention.

The transfer of foreign genes into the genome of a plant is termed transformation. In this process, the above-described methods of transforming and regenerating plants from plant tissues or plant cells are utilized for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic [sic] approach using the gene gun, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and Agrobacterium-mediated gene transfer. The methods mentioned are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, editors: S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus, Annu.Rev.Plant Physiol.Plant Molec.Biol. 42 (1991) 205-225). The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711).

Agrobacteria which have been transformed with an expression cassette according to the invention can then be used in the known manner for transforming plants, in particular crop plants such as cereals, maize, soya, rice, cotton, sugar beet, canola,

sunflower, flax, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various shrub, tree, nut and Vitis species, for example coffee, fruit trees such as apples, pears or cherries, nut trees such as walnut or pecan and, especially importantly, grapevines, for example by bathing wounded leaves or leaf sections in an agrobacterial solution and subsequently growing them in suitable media.

Functionally equivalent sequences which encode a fungicide-binding polypeptide are, in accordance with the invention, those sequences which still have the desired functions, despite a different nucleotide sequence. Thus, functional equivalents encompass naturally occurring variants of the sequences described herein, and also artificial nucleotide sequences, for example artificial nucleotide sequences which have been obtained by chemical synthesis and are adapted to the codon usage of a plant.

In particular, functional equivalent is to be understood as a natural or artificial mutation of an originally isolated sequence which encodes the fungicide-binding polypeptide, which mutation continues to show the desired function. Mutations encompass substitutions, additions, deletions, exchanges or insertions of one or more nucleotide residues. Thus, the present invention also encompasses those nucleotide sequences which are obtained by modifying this nucleotide sequence. The purpose of such a modification can be, for example, the further limitation of the encoding sequence contained therein, or else, for example, the insertion of more cleavage sites for restriction enzymes.

Other functional equivalents are those variants whose function is less or more pronounced, in comparison with the starting gene or gene fragment.

Moreover, artificial DNA sequences are suitable as long as they mediate the desired tolerance to fungicides for avoiding phytotoxic effects on crop plants, as described above. Such artificial DNA sequences can be identified, for example, by backtranslating proteins which have fungicide-binding activity and which have been constructed by means of molecular modeling, or by in vitro selection. Especially suitable are encoding DNA sequences which have been obtained by backtranslating a polypeptide sequence in accordance with the codon utilization which is specific to the host plant. The specific codon utilization can be determined readily by an expert familiar with methods of plant genetics by computer-aided evaluation of other,

known genes of the plant to be transformed.

Further suitable equivalent nucleic acid sequences according to the invention which must be mentioned are sequences which encode fusion proteins, where part of the fusion protein is a non-plant-derived fungicide-binding polypeptide or a functionally equivalent part thereof. For example, the second part of the fusion protein can be a further polypeptide with enzymatic activity, or an antigenic polypeptide sequence with the aid of which detection of scFvs expression is possible (for example myc-tag or his-tag). However, it is preferably a regulatory protein sequence, for example a signal or transit peptide, which directs the polypeptide with fungicide-binding properties to the desired site of action.

However, the invention also relates to the expression products produced in accordance with the invention and to fusion proteins of a transit peptide and a polypeptide with fungicide-binding properties.

Resistance/tolerance means, for the purposes of the present invention, the artificially acquired ability of plants to withstand fungicides with phytotoxic activity. It embraces the partial and, in particular, complete insensitivity to these inhibitors for the duration of at least one plant generation.

The phytotoxic site of action of fungicides is generally the leaf tissue, so that leaf-specific expression of the exogenous fungicide-binding polypeptide is capable of providing sufficient protection. However, one will understand readily that the phytotoxic action of a fungicide need not be restricted to the leaf tissue, but may also be effected in all remaining organs of the plant in a tissue-specific manner.

In addition, constitutive expression of the exogenous fungicide-binding polypeptide is advantageous. On the other hand, inducible expression may also be desirable.

The efficacy of the transgenically expressed polypeptide with fungicide-binding properties can be determined for example in vitro by shoot meristem propagation on fungicide-containing medium in series with staggered concentrations, or via seed germination tests. In addition, the fungicide tolerance, of a test plant, which has been altered with regard to type and level can be tested in greenhouse experiments.

The invention furthermore relates to transgenic plants, transformed with an expression cassette according to the invention, and to transgenic cells, tissues, organs and propagation material of such plants. Especially preferred are transgenic crop plants, for example cereals, maize, soya, rice, cotton, sugar beet, canola, sunflower, flax, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various shrub, tree, nut and Vitis species, for example coffee, fruit trees such as apples, pears or cherries, nut trees such as walnut or pecan, and, especially importantly, grapevine.

The transgenic plants, plant cells, plant tissues or plant organs can be treated with a fungicide with phytotoxic action which inhibits the plant enzymes, whereby the plants, plant cells, plant tissues or plant organs which have not been transformed successfully die or are damaged. Examples of suitable active ingredients are strobilurins, in particular methyl methoxyimino-α-(o-tolyloxy)-o-tolylacetate (BAS 490F), and metabolites and functional derivatives of these compounds. The DNA which encodes a polypeptide with fungicide-binding properties and which has been inserted into the expression cassettes according to the invention can thus also be used as selection marker.

The present invention has the advantage, in particular in the case of crop plants, that, once a selected resistance of the crop plant to fungicides with phytotoxic activity has been induced, such fungicides can be employed in these crops for controlling harmful fungi, even at higher rates of application which would otherwise lead to damaged plants. Compounds from the groups below may be mentioned as examples of such fungicides with phytotoxic activity, but not by way of limitation:

- sulfur, dithiocarbamates and their derivatives, such as iron(III) dimethyldithiocarbamate, zinc dimethyldithiocarbamate, zinc ethylenebisdithiocarbamate, manganese ethylenebisdithiocarbamate, manganese zinc ethylenediaminebisdithiocarbamate, tetramethylthiuram disulfides [sic], ammonia complex of zinc (N,N-ethylenebisdithiocarbamate), ammonia complex of zinc (N,N'-propylenebisdithiocarbamate), zinc (N,N'-propylenebisdithiocarbamate), N,N'-polypropylenebis(thiocarbamoyl)disulfide;
- nitro derivatives, such as dinitro(1-methylheptyl)phenyl crotonate, 2-sec-butyl-4,6-dinitrophenyl 3,3-dimethylacrylate, 2-sec-butyl-4,6-dinitrophenyl isopropyl carbonate, diisopropyl 5-nitroisophthalate;

- heterocyclic substances, such as 2-heptadecyl-2-imidazoline acetate, 2,4-dichloro-6-(o-chloroanilino)-s-triazine, O,O-diethyl phthalimidophosphonothioate, 5-amino-1-[bis(dimethylamino)phosphinyl]-3-phenyl-1,2,4-triazole, 2,3-dicyano-1,4-dithioanthraquinone, 2-thio-1,3-dithiolo[4,5-b]quinoxaline, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, 2-methoxycarbonylaminobenzimidazole, 2-(2-furyl)benzimidazole, 2-(4-thiazolyl)benzimidazole, N-(1,1,2,2-tetrachloroethylthio)tetrahydrophthalimide, N-trichloromethylthiotetrahydrophthalimide, N-trichloromethylthiophthalimide,
- N-dichlorofluoromethylthio-N',N'-dimethyl-N-phenylsulfodiamide, 5-ethoxy-3-trichloromethyl-1,2,3-thiadiazole, 2-thiocyanatomethylthiobenzothiazole, 1,4-dichloro-2,5-dimethoxybenzene, 4-(2-chlorophenylhydrazono)-3-methyl-5-isoxazolone, pyridine-2-thio [sic] 1-oxide, 8-hydroxyquinoline or its copper salt, 2,3-dihydro-5-carboxanilido-6-methyl-1,4-oxathiine, 2,3-dihydro-5-carboxanilido-6-methyl-1,4-oxathiine 4,4-dioxide, 2-methyl-5,6-dihydro-4H-pyran-3-carboxanilide, 2-methylfuran-3-carboxanilide, 2,5-dimethylfuran-3-carboxanilide, 2,4,5-trimethylfuran-3-carboxanilide, N-cyclohexyl-2,5-dimethylfuran-3-carboxamide, N-cyclohexyl-N-methoxy-2,5-dimethylfuran-3-carboxamide, 2-methylbenzanilide, 2-iodobenzanilide, N-formyl-N-morpholine-2,2,2-trichloroethyl acetal, piperazine-1,4-diylbis-1-(2,2,2-trichloroethyl)formamide, 1-(3,4-dichloroanilino)-1-formylamino-2,2,2-trichlorethane;
- amines such as 2,6-dimethyl-N-tridecylmorpholine or its salts, 2,6-dimethyl-N-cyclododecylmorpholine or its salts, N-[3-(p-tert-butylphenyl)-2-methylpropyl]-cis-2,6-dimethylmorpholine, N-[3-(p-tert-butylphenyl)-2-methylpropyl]piperidine, (8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine,
- azoles such as 1-[2-(2,4-dichlorophenyl)-4-ethyl-1,3-dioxolan-2-ylethyl]-1H-1,2,4-triazole, 1-[2-(2,4-dichlorophenyl)-4-n-propyl-1,3-dioxolan-2-ylethyl]-1H-1,2,4-triazole, N-(n-propyl)-N-(2,4,6-trichlorophenoxyethyl)-N'-imidazolylurea, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanol, (2RS,3RS)-1-[3-(2-chlorophenyl)-2-(4-fluorophenyl)-oxiran-2-

ylmethyl]-1H-1,2,4-triazole, 1-[2-(2,4-dichlorophenyl)-pentyl]-1H-1,2,4-triazole, 2,4'-difluoro-α-(1H-1,2,4-triazolyl-1-methyl)benzhydryl alcohol, 1-((bis(4-fluorophenyl)methylsilyl)methyl)-1H-1,2,4-triazole, 1-[2RS,4RS;2RS,4SR)-4-bromo-2-(2,4-dichlorophenyl)tetrahydrofuryl]-1H-1,2,4-triazole, 2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)-butan-2-ol, (+)-4-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether, (E)-(R,S)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pent-1-en-3-ol, 4-(4-chlorophenyl)-2-phenyl-2-(1H-1,2,4,-triazolylmethyl)butyronitrile, 3-(2,4-dichlorophenyl)-6-fluoro-2-(1H-1,2,4-triazol-1-yl)quinazolin-4(3H)-one, (R,S)-2-(2,4-dichlorophenyl)-1-H-1,2,4-triazol-1-yl)hexan-2-ol, (1RS,5RS;1RS,5SR)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol, (R,S)-1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol, (+)-2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazolyl)propyl 1,1,2,2-tetrafluoroethyl ether, (E)-1-[1-[4-chloro-2-trifluoromethyl)phenyl]imino)-2-propoxyethyl]-1H-imidazole, 2-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)hexanonitrile, α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol, 5-butyl-2-dimethylamino-4-hydroxy-6-methylpyrimidine, bis(p-chlorophenyl)-3-pyridinemethanol, 1,2-bis(3-ethoxycarbonyl-2-thioureido)benzene, 1,2-bis(3-methoxycarbonyl-2-thioureido)benzene,

- strobilurines such as methyl E-methoxyimino-[α-(o-tolyloxy)-o-tolyl]acetate, methyl E-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate, methyl-E-methoxyimino-[α-(2-phenoxyphenyl)]acetamide, methyl E-methoxyimino-[α-(2,5-dimethylphenoxy)-o-tolyl]acetamide,
- anilinopyrimidines such as N-(4,6-dimethylpyrimidin-2-yl)aniline, N-[4-methyl-6-(1-propynyl)pyrimidin-2-yl]aniline, N-[4-methyl-6-cyclopropylpyrimidin-2-yl]aniline,
- phenylpyrroles such as 4-(2,2-difluoro-1,3-benzodioxol-4-yl)pyrrole-3-carbonitrile,
- cinnamamides such as 3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)acryloylmorpholine,
- and a variety of fungicides such as dodecylguanidine acetate, 3-[3-(3,5-dimethyl-2-oxycyclohexyl)-2-hydroxyethyl]glutarimide, N-methyl-, N-ethyl-(4-trifluoromethyl, -2-[3',4'-dimethoxyphenyl]benzamide [sic], hexachlorobenzene, methyl N-(2,6-dimethylphenyl)-N-(2-furoyl)-DL-alaninate, DL-N-(2,6-dimethylphenyl)-N-(2'-methoxyacetyl)alanine methyl

ester, N-(2,6-dimethylphenyl)-N-chloroacetyl-D,L-2-aminobutyrolactone, DL-N-(2,6-dimethylphenyl)-N-(phenylacetyl)alanine methyl ester, 5-methyl-5-vinyl-3-(3,5-dichlorophenyl)-2,4-dioxo-1,3-oxazolidine, 3-[3,5-dichlorophenyl(-5-methyl-5-methoxymethyl]-1,3-oxazolidine-2,4-dione [sic], 3-(3,5-dichlorophenyl)-1-isopropylcarbamoylhydantoin, N-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide, 2-cyano-[N-(ethylaminocarbonyl)-2-methoximino]acetamide, N-(3-chloro-2,6-dinitro-4-trifluoromethylphenyl)-5-trifluoromethyl-3-chloro-2-aminopyridine.

Functionally equivalent derivatives of these fungicides have a comparable spectrum of action against phytopathogenic fungi such as the substances which have been mentioned specifically, combined with a less, equally or more pronounced phytotoxic activity.

The invention is now illustrated by the examples which follow, but is not limited thereto:

General cloning methods

The cloning steps carried out within the scope of the present invention, for example restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of E. coli cells, cultivation of bacteria, multiplication of phages and sequence analysis of recombinant DNA were carried out as described by Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6).

The bacterial strains used hereinbelow (E. coli, XL-I Blue) were obtained from Stratagene. The agrobacterial strain used for the transformation of plants (Agrobacterium tumefaciens, C58C1 with plasmid pGV2260 or pGV3850kan) was described by Deblaere et al. (Nucl. Acids Res. 13 (1985) 4777). Alternatively, the agrobacterial strain LBA4404 (Clontech) or other suitable strains may also be used. The vectors pUC19 (Yanish-Perron, Gene 33(1985), 103-119) pBluescript SK- (Stratagene), pGEM-T (Promega), pZerO (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12(1984) 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230) were employed for cloning purposes.

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using a laser fluorescence DNA sequencing apparatus from Pharmacia, using the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74(1977), 5463-5467).

Generation of plant expression cassettes

A 35S CaMV promoter was inserted into plasmid pBin19 (Bevan et al., Nucl. Acids Res. 12, 8711 (1984)) in the form of an EcoRI-KpnI fragment (corresponding to nucleotides 6909-7437 of the cauliflower mosaic virus (Franck et al. Cell 21 (1980) 285). The polyadenylation signal of gene 3 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835), nucleotides 11749-11939, was isolated in the form of a PvuII-HindIII fragment and, after SphI linkers had been added, cloned into the PvuII cleavage site between the SphI-HindIII cleavage site of the vector. This gave plasmid pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230).

Use Examples

Example 1

Since fungicides are not immunogenic, they must be coupled to a carrier material, for example KLH. If the molecule contains a reactive group, coupling may be effected directly; if not, a functional group is introduced when the fungicide is synthesized or a reactive precursor is selected during synthesis so as to couple these molecules to the carrier molecule in a simple reaction step. Examples of coupling reactions can be found in Miroslavic Ferencik in "Handbook of Immunochemistry", 1993, Chapman & Hall, in the chapter Antigens, pages 20 - 49.

Repeated injection of this modified carrier molecule (antigen) is used for immunizing, for example, Balb/c mice. Once a sufficient number of antibodies with binding to the antigen is detectable in the ELISA (enzyme-linked immunosorbent assay), the spleen cells of these animals are removed and fused with myeloma cells in order to cultivate hybrids. "Fungicide-modified BSA" is additionally used as antigen in the ELISA so as to differentiate the immune response directed against the hapten from the KLH response.

Monoclonal antibodies are prepared by methods similar to known methods, for example as described in "Practical Immunology", Leslie Hudson and Frank Hay, Blackwell Scientific Publications, 1989 or in "Monoclonal Antibodies: Principles and Practice", James Goding, 1983, Academic Press, Inc., or in "A practical guide to monoclonal antibodies", J.Liddell and A. Cryer,1991, John Wiley& Sons; or Achim Möller and Franz Emling "Monoklonale Antikörper gegen TNF und deren Verwendung" [Monoclonal antibodies against TNF, and their use]. European Patent Specification EP-A260610.

Example 2

The starting point of the investigation was a monoclonal antibody which specifically recognizes the fungicide BAS 490F and which, additionally, has a high binding affinity. The hydridoma cell line selected is characterized in that the secreted monoclonal antibodies which are directed against the fungicide antigen BAS 490F have a high affinity and the specific sequences of the immunoglobulins are available (Berek, C. et al., Nature 316 (1985), 412-418). This monoclonal antobidy against BAS 490F was the starting point for the construction of the single-chain antibody fragment (scFv-antiBAS 490F).

First, mRNA was isolated from the hybridoma cells and transcribed into cDNA. This cDNA acted as a template for the amplification of the variable immunglobulin genes VH and VK with the specific primers VH1 BACK and VH FOR-2 for the heavy chain and VK2 BACK and MJK5 FON X for the light chain (Clackson et al., Nature 352 (1991), 624-628). The variable immunoglobulins isolated were the starting point for the construction of a single-chain antibody fragment (scFv-antiBAS 490F). In the subsequent fusion PCR, three components VH,VK and a linker fragment were combined in a PCR reaction, and the scFv-antiBAS 490F was amplified (Fig. 3).

Functional characterization (antigen binding activity) of the scFv-antiBAS 490F gene constructed was carried out after expression in a bacterial system. To this end, the scFv-antiBAS 490F was synthesized in E. coli as a soluble antibody fragment, using the method of Hoogenboom, H.R. et al., Nucleic Acids Research 19 (1991), 4133-4137. Activity and specificity of the antibody fragment constructed were checked in an ELISA assay (Fig. 4).

To allow seed-specific expression of the antibody fragment in tobacco, the scFv-antiBAS 490F gene was cloned downstream from

the LeB4 promoter. The LeB4 promoter, which had been isolated from Vicia faba, shows strictly seed-specific expression of various foreign genes in tobacco (Bäumlein, H. et al., Mol. Gen. Genet. 225 (1991), 121-128). Transport of the scFv-antiBAS 490F polypeptide into the endoplasmatic reticulum resulted in stable accumulation of large amounts of antibody fragment. To this end, the scFv-antiBAS 490F gene was fused with a signal peptide sequence which guarantees entry into the endoplasmatic reticulum and with the ER retention signal SEKDEL, which guarantees that the polypeptide remains in the ER (Wandelt et al.,1992) (Fig. 5).

The expression cassette constructed was cloned into the binary vector pGSGLUC 1 (Saito et al., 1990) and transferred into the agrobacterium strain EHA 101 by electroporation. Recombinant agrobacterial clones were used for the subsequent transformation of Nicotiana tabacum. 70-140 tobacco plants were regenerated per construct. Seeds in different developmental stages were harvested from the regenerated transgenic tobacco plants, following self-pollination. The soluble proteins were obtained from these seeds in an aqueous buffer system, after extraction. Analysis of the transgenic plants demonstrates that fusion of the scFv-antiBAS 490F gene to the DNA sequence of the ER retention signal SEKDEL allowed a maximum accumulation of 1.9% scFv-antiBAS 490F protein to be obtained in the mature seed.

The scFv-antiBAS 490F gene constructed had a size of approximately 735 bp. The variable domains were fused to each other in the sequence VH-L-VL.

The specific selectivity was determined in the extracts of the mature tobacco seeds using a direct ELISA. The values obtained demonstrate clearly that the protein extracts contain functionally active antibody fragments.

Example 3

Seed-specific expression and concentration of single-chain antibody fragments in the endoplasmatic reticulum of cells of transgenic tobacco seeds, under the control of the USP promoter.

Starting point of the investigations was a single-chain antibody fragment against the fungicide BAS 490F (scFv-anti BAS 490F). The functional characterization (antigen binding activity) of this scFv-antiBAS 490F gene constructed was carried out following expression in a bacterial system and following expression in tobacco leaves. Activity and specificity of the antibody

fragment constructed were checked in an ELISA assay.

To allow seed-specific expression of the antibody fragment in tobacco, the scFv-antiBAS 490F gene was cloned downstream from the USP promoter. The USP promoter, which had been isolated from Vicia faba, shows strictly seed-specific expression of various foreign genes in tobacco (Fiedler, H. et al., Plant Mol. Biol. 22 (1993), 669-679). Transport of the scFv-antiBAS 490F polypeptide into the endoplasmatic reticulum resulted in stable accumulation of large amounts of antibody fragment. To this end, the scFv-antiBAS 490F gene was fused with a signal peptide sequence which guarantees entry into the endoplasmatic reticulum and with the ER retention signal SEKDEL, which guarantees that the polypeptide remains in the ER (Wandelt et al., 1992) (Fig. 1).

The expression cassette constructed was cloned into the binary vector pGSGLUC 1 (Saito et al., 1990) and transferred into the Agrobacterium strain EHA 101 by electroporation. Recombinant agrobacterial clones were used for the subsequent transformation of Nicotiana tabacum. Seeds in different developmental stages were harvested from the regenerated transgenic tobacco plants, following self-pollination. The soluble proteins were obtained from these seeds in an aqueous buffer system, after extraction. Analysis of the transgenic plants demonstrates that fusion of the scFv-antiBAS 490F gene to the DNA sequence of the ER retention signal SEKDEL under the control of the USP promoter caused single-chain antibody fragments with a binding affinity for BAS 490F to be synthesized as early as day 10 of the seed development.

Example 4

To achieve ubiquitous expression of the antibody fragment in the plant, especially in leaves, the scFv-antiBAS 490F gene was cloned downstream of the CaMV 35 S promoter. This strong constitutive promoter mediates expression of foreign genes in virtually all plant tissues (Benfey and Chua, Science 250 (1990), 956 - 966). Transport of the scFv-antiBAS 490F protein into the endoplasmatic reticulum allowed stable accumulation of large amounts of antibody fragment to be obtained in the leaf material. First, the scFv-antiBAS 490F gene was fused to a signal peptide sequence which ensures entry into the endoplasmatic reticulum and to the ER retention signal KDEL, which ensures that the product remains in the ER (Wandelt et al., Plant J. 2(1992), 181 - 192). The expression cassette constructed was cloned into the binary vector pGSGLUC 1 (Saito et al., Plant Cell Rep. 8(1990),718 -

721) and transferred into the Agrobacterium strain EHA 101 by electroporation. Recombinant agrobacterial clones were used for the subsequent transformation of Nicotiana tabacum. Approximately 100 tobacco plants were regenerated. Leaf material of various developmental stages was removed from the regenerated transgenic tobacco plants. The soluble proteins were obtained from this leaf material in an aqueous buffer system, following extraction. Subsequent analyses (western blot analyses and ELISA assays) demonstrated that a maximum accumulation of more than 2 % of biologically active antigen-binding scFv-antiBAS 490F polypeptide was obtained in the leaves. The high expression values were determined in fully grown green leaves, but the antibody fragment was also detected in senescent leaf material.

Example 5

PCR amplification of a fragment of the cDNA encoding the single-chain antibody against BAS 490F with the aid of synthetic oligonucleotides.

The PCR amplification of the single-chain antibody cDNA was carried out in a DNA thermal cycler from Perkin Elmer. The reaction mixtures contained 8 ng/µl single-stranded template cDNA, 0.5 µM of the relevant oligonucleotides, 200 µM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM MgCl2) and 0.02 U/µl Taq polymerase (Perkin Elmer). The amplification conditions were set as follows:

Annealing temperature:	45°C
Denaturation temperature:	94°C,
Elongation temperature:	72°C,
Number of cycles:	40

The result is a fragment of approx. 735 base pairs, which was ligated into the vector pBluescript. The ligation mixture was used for transforming E. coli XL-I Blue, and the plasmid was amplified. Regarding use and optimization of polymerase chain reaction, see: Innis et al., 1990, PCR Protocols, A Guide to Methods and Applications, Academic Press.

Example 6

Production of transgenic tobacco plants which express a cDNA encoding a single-chain antibody with fungicide-binding

properties.

Plasmid pGSGLUC 1 was transformed into Agrobacterium tumefaciens C58C1:pGV2260. To transform tobacco plants (Nicotiana tabacum cv. Samsun NN), a 1:50 dilution of an overnight culture of a positively transformed agrobacterial colony in Murashige-Skoog medium (Physiol. Plant. 15 (1962) 473 et seq.) containing 2% of sucrose (2MS medium) was used. In a Petri dish, leaf disks of sterile plants (each approx. 1 cm^2) were incubated for 5-10 minutes in a 1:50 agrobacterial dilution. This is followed by 2 days' incubation in the dark at 25°C on 2MS medium containing 0.8% Bacto-Agar. Cultivation was continued after 2 days in 16 hours light/8 hours dark and continued in a weekly rhythm on MS medium containing 500 mg/l Claforan (cefotaxim-sodium), 50 mg/l kanamycin, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid and 1.6 g/l glucose. Growing shoots were transferred to MS medium containing 2% sucrose, 250 mg/l Claforan and 0.8% Bacto-Agar.

Example 7

Stable accumulation of the single-chain antibody fragment against the fungicide BAS 490F in the endoplasmatic reticulum.

Starting point of the investigations was a single-chain antibody fragment against the fungicide BAS 490F(scFv-anti BAS 490F) which is expressed in tobacco plants. Quantity and activity of the scFv-antiBAS 490F polypeptide synthesized were determined in western blot analyses and ELISA assays.

To make possible expression of the scFv-antiBAS 490F gene in the endoplasmatic reticulum, the foreign gene was expressed under the control of the CaMV 53S promoter as a translation fusion with the LeB4 signal peptide (N-terminal) and the ER retention signal KDEL (C-terminal). Transport of the scFv-antiBAS 490F polypeptide into the endoplasmatic reticulum allowed stable accumulation of large quantities of active antibody fragment. After the leaf material had been harvested, sections were frozen at -20°C (1), lyophilized (2) or dried at room temperature (3). The soluble proteins were obtained from the leaf material in question by extraction in an aqueous buffer, and the scFv-antiBAS 490F polpypeptide was purified by affinity chromatography. Equal amounts of purified scFv-antiBAS 490F polypeptide (frozen, lyophilized and dried) were employed for determining the activity of the antibody fragment (Fig. 6). Fig. 6A shows the antigen binding activity of the scFv-antiBAS 490F polypeptide purified from fresh (1),

lyophilized (2) and dried leaves (3). In Fig. 6B, the respective amounts of scFv-antiBAS 490F protein (approx. 100 ng) which were employed for the ELISA analyses are determined by means of Western blot analyses. The sizes of the protein molecular weight standards are shown on the left. Approximately identical antigen binding activities were found.

Example 8

To demonstrate the fungicide tolerance of the transgenic tobacco plants which produce a polypeptide with fungicide-binding properties, these tobacco plants were treated with various amounts of BAS 490F. It was possible to demonstrate in all cases, in the greenhouse, that the plants expressing a scFv-antiBAS 490F gene showed a higher tolerance to the fungicide BAS 490F and less pronounced phytotoxic effects in comparison with the control.

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We claim:

1. A process for the production of a fungicide-tolerant plant by expressing an exogenous fungicide-binding polypeptide in the plant.

2. A process as claimed in claim 1, wherein the exogenous fungicide-binding polypeptide is a single-chain antibody fragment.

3. A process as claimed in claim 1, wherein the exogenous fungicide-binding polypeptide is a complete antibody or a fragment derived therefrom.

4. A process as claimed in claim 1, wherein the fungicide is methyl methoxyimino-α-(o-tolyloxy)-o-tolylacetate (BAS 490F).

5. A process as claimed in any of claims 1 - 3, wherein the plant is mono- or dicotyledonous.

6. A process as claimed in claim 5, wherein the plant is tobacco.

7. A process as claimed in any of claims 1 - 6, wherein the exogenous polypeptide is expressed constitutively in the plant.

8. A process as claimed in any of claims 1 - 6, wherein expression of the exogenous polypeptide in the plant is induced.

9. A process as claimed in any of claims 1 - 6, wherein the exogenous polypeptide is expressed in the leaves of the plant.

10. A process as claimed in any of claims 1 - 6, wherein the exogenous polypeptide is expressed in the seeds of the plant.

11. An expression cassette for plants, composed of a promoter, a signal peptide, a gene encoding expression of an exogenous fungicide-binding polypeptide, an ER retention signal and a terminator.

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12. An expression cassette as claimed in claim 11, wherein the constitutive promoter used is the CaMV 35S promotor.

13. An expression cassette as claimed in claim 11, wherein the gene to be expressed is the gene of a single-chain antibody fragment.

14. An expression cassette as claimed in claim 11, wherein the gene or gene fragment of a fungicide-binding polypeptide in the form of a translation fusion with other functional proteins, for example enzymes, toxins, chromophores and binding proteins, is employed as the gene to be expressed.

15. An expression cassette as claimed in claim 11, wherein the polypeptide gene to be expressed is obtained from a hybridoma cell or with the aid of other recombinant methods, for example the antibody phage display method.

16. The use of the expression cassette as claimed in claim 11 for the transformation of dicotyledonous or monocotyledonous plants which constitutively express an exogenous fungicide-binding polypeptide seed- or leaf-specifically.

17. The use as claimed in claim 16, wherein the expression cassette is transferred into a bacterial strain and the resulting recombinant clones are used for the transformation of the dicotyledonous or monocotyledonous plants which constitutively express an exogenous fungicide-binding polypeptide seed- or leaf-specifically.

18. The use of the expression cassette as claimed in claim 11 as selection marker.

19. The use of a transformed plant as obtained in accordance with claim 17 or 18 for the production of a fungicide-binding polypeptide.

20. A process for the transformation of a plant by introducing a gene sequence which encodes a fungicide-binding polypeptide into a plant cell, into callus tissue, an entire plant and protoplasts of plant cells.

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21. A process as claimed in claim 20, wherein transformation is effected with the aid of an agrobacterium, in particular of the species Agrobacterium tumefaciens.

22. A process as claimed in claim 20, wherein transformation is effected with the aid of electroporation.

23. A process as claimed in claim 20, wherein transformation is effected with the aid of the particle bombardment method.

24. The production of a fungicide-binding polypeptide by expressing a gene which encodes such a polypeptide in a plant or cells of a plant and subsequently isolating the polypeptide.

25. A plant comprising an expression cassette as claimed in claim 11, wherein the expression cassette imparts tolerance to a fungicide.

26. A plant as claimed in claim 25 which is tolerant to methyl methoxyimino-α-(o-tolyloxy)-o-tolylacetate (BAS 490F).

27. A method of controlling phytopathogenic fungi in transgenic fungicide-tolerant crop plants, which comprises the use of fungicides against which the crop plant forms fungicide-binding polypeptides or antibodies.

28. A fungicide-binding polypeptide or antibody with high binding affinity to methyl methoxyimino-α-(o-tolyloxy)-o-tolylacetate (BAS 490F) which is produced as claimed in claim 24.

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Expression of fungicide-binding polypeptides in plants for generating fungicide tolerance

Abstract

Processes for the production of fungicide-tolerant plants by expressing a fungicide-binding antibody in the plants.

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