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WO-A-97/49277 US-P-6 166

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- MAURO M C ET AL: "High efficiency regeneration of grapevine plants transformed with the GFLV coat protein gene" PLANT SCIENCE (LIMERICK), vol. 112, no. 1, 1995, pages 97-106, XP001189302 ISSN: 0168-9452
- BARDONNET et al., "Protection Against Virus Infection in Tobacco Plants Expressing the Coat Protein of Grapevine Fanleaf Nepovirus", PLANT CELL REP., 1994, Vol. 13, pages 357-360, XP002915219

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Remarks:
The file contains technical information submitted after the application was filed and not included in this specification
This invention relates to a method for selecting and producing a transgenic grapevine or grapevine component having increased resistance to a fanleaf disease.

Grapevine fanleaf virus (GFLV) is a grape nepovirus, which is transmitted from plant to plant by the dagger nematode, Xiphinema index. GFLV is the agent responsible for grapevine fanleaf disease, which occurs worldwide. The disease is named for the fan-leaf shaped appearance of GFLV-infected leaves. It is one of the most damaging and widespread diseases of grapevine. Symptoms of GFLV infection include abnormal shoot morphology and discolorations of the leaves, yielding a fan-like appearance (Agrios, Plant Pathology, 3rd Edition, Academic Press, 1988, pp. 687-688). In addition, fruit production of infected vines is low, with grapevines producing small bunches having abnormal fruit set and ripening. Ultimately, infected grapevines degenerate and die.

Long range spread of GFLV is believed to be by use of infected planting material. While the natural host range is thought to be restricted to grape, GFLV is also transmissible to a wide range of herbaceous species by sap-rubbing inoculation. Chenopodium quinoa is a useful diagnostic species for the virus. In general, GFLV isolates are antigenically uniform and diagnosis by ELISA is a standard procedure.

Current strategies for controlling grapevine fanleaf disease and other nepovirus-induced diseases in vineyards include nematode control (for example, soil fumigation and use of other pesticides), breeding rootstocks for resistance to nematode feeding, breeding grapevines for resistance to GFLV, and planting certified disease-free grapevines.

In general, the invention features a method for producing and selecting a transgenic grapevine or grapevine component having increased resistance to a fanleaf disease as set out in claim 1. The method generally involves: (a) transforming a grape plant cell with a grape nepovirus coat protein nucleic acid molecule or fragment thereof (for example, a grape nepovirus coat protein nucleic acid molecule or fragment thereof having about 50% or greater sequence identity to SEQ ID NO: 1) which is capable of being expressed in a plant cell; (b) regenerating a transgenic grapevine or grapevine component from the plant cell; and (c) selecting a transgenic grapevine or grapevine component which expresses the nucleic acid molecule or fragment thereof at a level that is negative for expression of grape nepovirus coat protein or fragment thereof as detected by ELISA, wherein the expression increases the resistance of the transgenic grapevine or grapevine component to fanleaf disease as compared to plants expressing the nucleic acid molecule at a high level.

Low level expression of the grape nepovirus mRNA or of the expressed coat protein itself in the transgenic plant is measured by ELISA and inoculation of transgenic plants with virus and selection of resistant vines. In preferred embodiments, the nucleic acid molecule or fragment thereof is encoded by a transgene found in the transgenic grapevine. The nucleic acid molecule or fragment thereof is expressed as in a sense orientation. In yet other preferred embodiments, such grape nepovirus coat protein nucleic acid molecules or fragments thereof is expressed in the transgenic grapevine or grapevine component.

As is discussed above, the invention also includes fragments of a grape nepovirus coat protein nucleic acid molecule that facilitate, when expressed at a level that is negative for expression of grape nepovirus coat protein or fragment thereof as detected by ELISA, an increased resistance of a transgenic grapevine or grapevine component thereof, to a fanleaf disease. Thus, grape nepovirus coat protein nucleic acid sequences described herein or portions thereof may be expressed in a plant to facilitate disease resistance. Sequences that mediate an increased resistance to a fanleaf disease are considered useful in the invention. As used herein, the term "fragment," as applied to sequences of a nucleic acid molecule, means at least 5 contiguous nucleotides, preferably at least 10 contiguous nucleotides, more preferably at least 20 to 30 contiguous nucleotides, and most preferably at least 40 to 80 or more contiguous nucleotides. Fragments of a grape nepovirus nucleic acid molecule can be produced and, subsequently, integrated into any standard expression vector (for example, those described herein) according to methods known to those skilled in the art.

Preferably, the grapevine useful in the invention is a member of the genus Vitis; and the grapevine component is a somatic embryo, a scion, a rootstock, or a mother block. The fanleaf disease is grapevine fanleaf disease caused by a grape nepovirus. In yet other preferred embodiments, the grape nepovirus is a grapevine fanleaf virus or an arabis mosaic virus.

The methods and GFLV sequences described herein are useful for providing disease resistance or tolerance or both on a variety of grapevines (for example, Vitis spp., Vitis spp. hybrids, and all members of the subgenera Euvitis and Muscadinias), including scion or rootstock cultivars. Exemplary scion cultivars include, without limitation, those which are referred to as table or raisin grapes and those used in wine production such as Cabernet Franc, Cabernet Sauvignon, Chardonnay (for example, CH 01, CH 02, CH Dijon), Merlot, Pinot Noir (PN, PN Dijon), Semillon, White Riesling, Lambrusco, Thompson Seedless, Autumn Seedless, Niagara Seedless, and Seval Blanc. Rootstock cultivars that are
useful in the invention include, without limitation, Vitis rupestris Constantia, Vitis rupestris St. George, Vitis californica,
Vitis girdiana, Vitis rotundifolia, Vitis rotundifolia Carlos, Richter 110 (Vitis berlandieri x rupestris), 101-14 Millioner
de Grasset (Vitis riparia x rupestris), Teleki SC (Vitis berlandieri x riparia), 3309 Courderc (Vitis riparia x rupestris),
riparia Gloire de Montpellier (Vitis riparia), SBB Teleki (selection Kober, Vitis berlandieri x riparia), SO4 (Vitis berlandieri
x rupestris), 41B Millioner (Vitis vinifera x berlandieri), and 039-16 (Vitis vinifera x Muscadinia).

[0009] By "nontranslatable" is meant an mRNA sequence that is not translated into a protein. Examples of such
nontranslatable sequences include, without limitation, sequences including an initiation ATG codon followed by an
engineered frameshift mutation and stop codon to prevent translation of the mRNA into a protein. Grape nepovirus
coop protein genes expressing such nontranslatable mRNA sequences may be constructed according to standard methods
(for example, those described herein).

[0010] The method for analyzing expression of a grape nepovirus coat protein gene is the immunological technique
ELISA for detection of a protein.

[0011] By "substantially identical" is meant a protein or nucleic acid molecule exhibiting at least 97%, and preferably
98%, or more preferably 99% identity to a reference amino acid sequence (for example, the amino acid sequence shown
in Fig. 1; SEQ ID NO: 2) or nucleic acid sequence (for example, the nucleic acid sequences shown in Fig. 1; SEQ ID
NO: 1). For proteins, the length of comparison sequences will generally be at least 16 amino acids, preferably at least
20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids or greater. For nucleic
acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides,
more preferably at least 75 nucleotides, and most preferably 110 nucleotides or greater.

[0012] Sequence identity, at the amino acid or nucleic acid levels, is typically measured using sequence analysis
software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin
Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs). Such
software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions,
and/or other modifications. Conservative amino acid substitutions typically include substitutions within the following
groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine;
lysine, arginine; and phenylalanine, tyrosine.

[0013] By a "substantially pure protein" is meant a grape nepovirus coat protein (for example, the coat protein from
the Geneva, N.Y. grape nepovirus isolate (Fig. 1; SEQ ID NO: 2)) that has been separated from components which
naturally accompany it. Typically, the protein is substantially pure when it is at least 60%, by weight, free from the proteins
and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least
75%, more preferably at least 90%, and most preferably at least 99%, by weight, protein. A substantially pure protein
(for example, the coat protein of the 'Geneva' grape nepovirus isolate) may be obtained, for example, by extraction from
a natural source (for example, a GFLV-CP infected plant such as C. quinoa); by expression of a recombinant nucleic
acid encoding a protein; or by chemically synthesizing the protein. Purity can be measured by any appropriate method,
for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

[0014] By "isolated nucleic acid molecule" is meant a nucleic acid molecule (for example, DNA) that is free of the
nucleic acids which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the
invention is derived, flank the nucleic acid molecule. The term therefore includes, for example, a recombinant DNA that
is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote
or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by
PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which
is part of a hybrid gene encoding additional protein sequence.

[0015] By "specifically hybridizes" is meant that a nucleic acid molecule that is capable of hybridizing to a nucleic acid
sequence (for example, DNA) at least under low stringency conditions, and preferably under high stringency conditions.

[0016] By "protein" is meant any chain of amino acids, including polypeptides, regardless of length or post-translational
modification (for example, glycosylation or phosphorylation), including polypeptides.

[0017] By "positioned for expression" is meant that the nucleic acid molecule (for example, DNA) is positioned adjacent
to a sequence which directs transcription of the nucleic acid molecule.

[0018] By "expression control region" is meant any minimal sequence sufficient to direct transcription. Included in the
invention are promoter and enhancer elements that are sufficient to render promoter-dependent gene expression con-
trollable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents
(for example, light-, pathogen-, wound-, stress- or hormone-inducible elements; or constitutive elements); such elements
may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

[0019] By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way to permit
gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the
regulatory sequence(s).

[0020] By "plant cell" is meant any self-propagating cell bounded by a semipermeable membrane and containing a
plastid. A plant cell, as used herein, is obtained from, without limitation, seeds, suspension cultures, embryos, meris-
Detailed Description

By "plant component" is meant a part, segment, or organ obtained from an intact plant or plant cell. Exemplary plant components include, without limitation, somatic embryos, leaves, fruits, scions and rootstocks.

By "transgenic" is meant any cell which includes a nucleic acid molecule (for example, a DNA sequence) which is inserted by artifice into a cell and becomes part of the organism (in either an integrated or extrachromosomal fashion for example, a viral expression construct which includes a subgenomic promoter) which develops from that cell. Such a transgenic may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "increased resistance to a fanleaf disease" is meant a greater level of resistance to a fanleaf disease (for example, any disease caused by a grape nepovirus such as those caused by GFLV, arabis mosaic virus, and the like) in a transgenic grapevine (or grapevine component or cell or seed thereof) than the level of resistance relative to a non-transgenic grapevine. In preferred embodiments, the level of resistance in a transgenic grapevine is at least 5 to 10% (and preferably 20%, 30%, or 40%) greater than the resistance of a control grapevine. In other preferred embodiments, the level of resistance to fanleaf disease is 50% greater, 60% greater, and more preferably even 75% or 90% greater than a control grapevine; with up to 100% resistance as compared to a control grapevine being most preferred. The level of resistance is measured using conventional methods. For example, the level of resistance to fanleaf disease may be determined by comparing physical features and characteristics (for example, plant height and weight, or by comparing disease symptoms, for example, delayed lesion development, reduced lesion size, leaf wilting and curling, mottling and necrosis of leaves, deformity of canes, number of internodes, mosiac rings on leaves, and discoloration of cells) of transgenic grapevines. Infectivity of a grape nepovirus (for example, a GFLV or an arabis mosaic virus) can also be monitored using, for example, standard ELISA.

By "transgenic" is meant any piece of a nucleic acid molecule (for example, DNA) which is inserted by artifice into a cell, and becomes part of the organism (integrated into the genome or maintained extrachromosomally) which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgene" is meant any piece of a nucleic acid molecule (for example, DNA) which is inserted by artifice into a cell and becomes part of the organism (integrated into the genome or maintained extrachromosomally) which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

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By "transgenic" is meant any cell which includes a nucleic acid molecule (for example, a DNA sequence) which is inserted by artifice into the nuclear or plastidic compartments of the plant cell. Such transgenic grapevine or grapevine component express at least one sense translatable sequence grape nepovirus transcript.

By "transgenic" is meant any piece of a nucleic acid molecule (for example, DNA) which is inserted by artifice into a cell, and becomes part of the organism (integrated into the genome or maintained extrachromosomally) which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

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Drawings

Fig. 1 is a schematic illustration showing the nucleotide (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the coat protein of a Geneva, N.Y. grape nepovirus isolate.

Fig. 2 is a schematic illustration showing the maps of the plant expression vectors containing different viral gene constructs.
Fig. 3 shows the results of experiments analyzing the expression levels of grapevine fanleaf coat protein in transgenic 3309C and Gloire.

A description for the production of disease resistant transgenic grapevines now follows. Transgenic grape plants expressing either sense translatable of grapevine fanleaf virus coat protein (GFLV-CP) genes were regenerated from embryogenic callus cultures derived from anthers of rootstocks (3309 Couderc ("3309C"), Riparia Gloire ("Gloire"), Teleki 5C ("5C"), 110 Richter ("110R"), S04, and MGT 101-14 ("101-14")). Unexpectedly, transgenic plants expressing low levels of a recombinant grapevine fanleaf coat protein gene were found to be resistant to fanleaf disease.

The examples provided below are for the purpose of illustrating the invention, and should not be construed as limiting.

Results

Initiation of Embryogenic Callus and Embryogenesis

Callus was initiated from grape cultivars: Gloire, 3309C, 5C, 110R, and 101-14 on MSE medium (infra). Anthers from flower buds of the five rootstocks began to swell after one week in culture. After four weeks, a smooth, gelatinous, bright yellow callus developed. At this time, some embryos of Gloire, 3309C, and 110R were visible on the callus tissue. After eight weeks, all the calli were transferred to HMG medium (infra) to permit further development of the embryos. By the eighth week on HMG medium, many embryo clusters were induced from the callus tissue.

Plant Regeneration

After cultivation for eight to sixteen weeks in HMG medium, embryo clusters, and hypocotyls were found to develop from the calli. At the same time, secondary embryos were continually produced from the primary embryos. Embryo clusters were next transferred to MGC medium (infra) to increase embryo size and growth rate. However, fewer embryos were produced on MGC medium as compared to HMG medium for all rootstock cultivars that were examined. Embryo development of cultivar 110R was found to be dependent on the use of both media; HMG medium was required to induce many small secondary embryos and MGC medium was needed to simulate hypocotyl growth.

Hypocotyls were subsequently transferred onto a woody plant medium (Lloyd and McCown, infra) and shoots appeared within one to two months. The plantlets were generally induced at thirty to sixty-six frequency on woody plant medium.

The resulting plantlets were then transplanted to soil and kept in the greenhouse. Plants of Gloire, 5C, 110R, 101-14, and 3309C exhibited normal morphology.

Maintenance of Somatic Embryogenesis

A continuous supply of embryogenic calli were produced using an embryo cycling method; pieces of hypocotyl induced embryogenic callus on the MSE medium in two to three months. These calli were amenable for transformation because they developed many uniform embryos. Embryos of 5C required culture on MSE medium for three months, followed by culture on HMG medium for two to three months to induce the formation of embryos. The duration of time required for embryo cycling (embryogenic callus to hypocotyl and back to embryogenic callus) varied for the different cultivars; Riparia Gloire required two to three months, 3309C and 101-14 required five to six months, and 5C required six to seven months.

Transformation

Using standard techniques of molecular biology, a nucleotide sequence encoding a coat protein gene of a Geneva, NY grape nepovirus isolate was isolated and characterized. The nucleic acid sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the ‘Geneva’ isolate coat protein gene are shown in Fig. 1. These sequences were also compared to three different GFLV isolates from France (Serghini et al., J. Gen. Virol. 71: 1433-1442, 1990), California (Sanchez et al., Nucleic Acids Res. 19 5440, 1991), and Austria (Brandt et al., Arch. Virol. 140: 157-164, 1995) using the Prettybox program. The percentage of identity of the amino acid sequences of the French, Californian, and Austrian isolates with the ‘Geneva’ isolate was 96.4%, 95.0%, and 95.4%, respectively.

Three different gene constructs (sense translatable, sense nontranslatable, and antisense) were used to transform grape (Fig. 2). Small somatic embryos and embryogenic calli of the five rootstocks Gloire, 3309C, 110R, 5C, and 101-14 were cocultivated with A. tumefaciens strain C582707 harboring binary vectors carrying the coat protein gene of the ‘Geneva’ isolate. After cocultivation, somatic embryos were transferred to HMG or MSE media with cefotaxime,
carbenicillin, and kanamycin to select transgenic embryos and plants. Transgenic plants were thus generated.

Analysis of Transgenic Plants

[0038] In an experiment with a GFLV-CP sense-translatable and β-glucuronidase gene expression constructs (FLcpST+GUS, Fig. 2), putative transgenic plants of Gloire and of 110R were assayed for GUS activity, the NPTII gene, and expression of the GFLV-CP gene by ELISA. By PCR analysis, about 93% of the transformed Gloire plants were found to have the GFLV-CP gene of the expected size, yet these plants were negative for GFLV expression by ELISA. [0039] These results indicated that GFLV-CP gene expression in the Gloire transgenics was too low to detect using ELISA. In contrast cultivar 3309C was transformed with GFLV-CP sense-translatable construct in a vector without GUS. We analyzed coat protein expression of putative transgenic plants, and found that 97.7% ELISA positive plants. Among these plants, 37.7% showed low expression (0.1<OD 405 >0.5), 30.7% showed medium expression (0.5<OD 405 >1.0), and 31.0% had high expression (OD 405 >1.0). Nontransformed control plants were negative (OD 405 <0.020).

[0040] In another series of experiments, ELISA results also revealed different levels of expression of GFLV coat protein gene in transgenic plants of 3309C and Gloire. Low coat protein gene expression was observed in 61 % and 53% of the transformed 3309C and Gloire, respectively. Medium coat protein gene expression was found in 26% and 22%, respectively. High coat protein gene expression was found in 13% and 25%, respectively, of the transformed 3309C and Gloire (Fig. 3).

Protection Against GFLV Infection

[0041] Transgenic plants were tested for GFLV resistance as follows. Plants were inoculated with GFLV by hetero-grafting to GFLV-infected C. quinoa or by grafting to nontransgenic GFLV-infected cultivars according to standard methods. The plants were maintained for several months after inoculation and then were evaluated for disease resistance. Disease resistance was assessed by standard ELISA. The results of these experiments are shown in Tables I-V (below). In particular, as is shown in Table III, one transgenic line expressing the antisense expression constructs GFLVcpAntiS (Fig. 2) used for comparison was found to resist GFLV infection.

### Table I

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>ELISA Evaluation of Transgenic 110 Richter Heterografted with Infected C. quinoa</th>
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<tr>
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<td>ELISA 4/31/97</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>#84</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
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<sup>1</sup>FLcpST+GUS (Fig. 2); NT: Not tested

### Table II

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>ELISA Evaluation of Transgenic Riparia Gloire Heterografted with GFLV Infected C. quinoa</th>
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<tr>
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(continued)

### Table III

**ELISA Evaluation of Transgenic Riparia Gloire Heterografted with GFLV Infected *C. quinoa***

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<th>4-5 months</th>
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<th>(%) infected</th>
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<td>23/26</td>
<td>23/26</td>
<td>23/26</td>
<td>88.0</td>
</tr>
</tbody>
</table>

1 FLcpST+GUS (Fig. 2)

### Table IV

**Micrografting In vitro**

<table>
<thead>
<tr>
<th>Transgenic L./ GFLV grape</th>
<th>Number of plantlets in GH</th>
<th>ELISA 11/27/96</th>
<th>ELISA 1/30/97</th>
<th>ELISA 4/24/97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riparia # 16/Cabernet sauv.</td>
<td>5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Riparia # 21/Cabernet sauv.</td>
<td>5</td>
<td>1/5</td>
<td>1/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Riparia # 23/Cabernet sauv.</td>
<td>5</td>
<td>2/5</td>
<td>2/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Riparia # 19/Cabernet sauv.</td>
<td>4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Riparia B-2/Cabernet sauv.</td>
<td>4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Riparia B-13/Cabernet sauv.</td>
<td>4</td>
<td>1/4</td>
<td>2/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Riparia B-17/Cabernet sauv.</td>
<td>2</td>
<td>1/2</td>
<td>1/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Riparia B-67/Cabernet sauv.</td>
<td>2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Riparia, ht/Cab</td>
<td>5</td>
<td>4/5</td>
<td>4/5</td>
<td>4/4</td>
</tr>
</tbody>
</table>

1 FLcpAntiS (Fig. 2); 2 FLcpST (Fig. 2) used for comparison
Materials and Methods

The above-described results were carried out using the following materials and methods.

Plant materials

The rootstock cultivars Couderc 3309 ("3309C") (V. riparia x V. rupestris), Riparia Gloire ("Gloire") (V. riparia), Teleki 5C ("5C") (V. berlandieri x V. riparia), MGT 101-14 ("101-14") (V. riparia x V. rupestris) and 110 Richter ("110R") (V. rupestris x V. berlandieri) were used in the above-described experiments. Callus cultures were initiated from anthers using the methods of Rajasekaram and Mullins (J. Exp. Bot. 30: 399-407, 1979). Flower buds of 3309C, 5C, 110R and 101-14 were collected from a vineyard at the Geneva Experiment Station, Geneva, N.Y. Gloire dormant canes were collected from the same vineyard and stored in moist perlite in plastic bags at 4°C. Two to five node sections were rooted in pots with perlite in the greenhouse; floral buds developed within four weeks. Flower buds were harvested prior to anthesis from field-grown vines. Buds were removed from the clusters and surface sterilized in 70% ETOH for one to two minutes. The buds were transferred to 1% sodium hypochlorite for fifteen minutes, then rinsed three times in sterile double-distilled water. Anthers were excised aseptically from flower buds while using a stereo microscope. To determine which state was most favorable for callus induction, the pollen was crushed on a microscope under a coverslip with a drop of acetocarmine to observe the cytological stage according to standard methods.

Media

Four different solid media were used to produce embryos and regenerate plants. The four media used are as follows. (1) Initiation medium. This medium was an amended MS medium (Murashige and Skoog, Physiol. Plant. 15: 473-497, 1962) and is referred to as MSE (Mozsar and Sule, Vitis 33: 245-246, 1994). (2) Differentiation medium. This medium is referred to as HMG medium as described by Mozsar and Sule (Vitis 33: 245-246, 1994); (3) Regeneration medium. This medium is refereed to as MGC medium. It is composed of full-strength MS salts amended with 20 g/L sucrose, 4.6 g/L glycerol, 1 g/L casein hydrolysate and 0.8% Noble agar; and (4) Rooting medium. This medium (pH 5.8) is woody plant medium (Lloyd and McCown, Proc. Intl. Plant Prop. Soc. 30: 421-427, 1981) supplemented with 0.1 mg/L BA, 3 g/L activated charcoal and 1.5% sucrose.

Somatic Embryogenesis and Regeneration

Anthers were isolated under sterile conditions and plated at a density of forty to fifty anthers per 9.0 cm diameter Petri dish and cultured at 28°C in the dark. Callus was induced on MSE. After sixty days, embryos were induced and then transferred to hormone-free HMG medium for differentiation. Torpedo-stage embryos were transferred from HMG to MGC medium to promote embryo germination. Cultures were maintained in the dark at 26-28°C and transferred to fresh medium at three to four week intervals. Hypocotyls (elongated embryos) were transferred to rooting medium in baby food jars (five to eight embryos per jar). The embryos were grown at 25°C with a daily sixteen hour photoperiod to induce shoot and root formation. After root development plants were transplanted to soil and placed in the greenhouse.
Maintenance and Propagation of Somatic embryos

[0046] Hypocotyls from elongated embryos that developed in HMG or MGC medium were cut into 3-4 mm pieces and placed onto MSE medium to promote the development of secondary embryogenic calli. The secondary embryogenic calli were then transferred to HMG for differentiation and development of new hypocotyls. These secondary hypocotyls from HMG medium were then transferred to MSE medium to obtain a third cycle of embryogenic calli and hypocotyls. The fourth and fifth cycles of embryogenic calli were obtained in a similar manner. Alternatively, embryogenic calli developing from the anthers were propagated on MSE medium to produce sufficient young embryos for transformation. All embryo cultures were transferred at twenty to thirty day intervals to fresh medium for maintenance.

Genes and Vectors

[0047] Three genetic constructs were used to genetically transform grape in this study (Fig. 2). A. tumefaciens strain C58Z707 containing either the binary plasmid pGA482GG or pGA482G were used to transform the grape plants with GFLV-cp. The coat protein gene of a GFLV designated as ‘Geneva’ NY isolate CF57 was cloned and sequenced according to standard methods. The coat protein of grapevine fanleaf nepovirus is produced by posttranslational processing of the polyprotein by virus encoded proteinase. The coat protein gene, which is located on the 3’ half of the RNA2 genome, does not contain an ATG initiation codon. Oligonucleotide primers containing the Ncol site were therefore used to introduce the translatable initiation codon into a genetic construct. Two primer sets designed to flank the coat protein gene for PCR amplification were utilized according to standard methods. The primer set (P2: agtgctCTCGAG restriction sites which were used for efficient cloning. Bold letters represent the stop codon which was used to engineer (Lower case letters are nonsense sequences which are utilized for effective restriction digestion. Underlined areas are specific primer (KSL95-10: ctgtaCCATGG + TCTTT- CAATTGAGACTTTTCAACAA; SEQ ID NO: 6) and transgene primers. The expression cassette containing the coat protein gene for PCR amplification were utilized according to standard methods. The primer set (P2: cgtcagTCTAGACCATGGTCTTT-CAATTGAGACTTTTCAACAA; SEQ ID NO: 4) was used to generate a translatable construct. To engineer a sense nontranslatable construct, we introduced an additional nucleotide (T) only three nucleotides downstream of the translational initiation codon (ATG) to make a frameshift mutation, as well as to create a stop codon. This was accomplished using the KSL95-10 (SEQ ID NO: 4) and KSL96-15 (acgttaCCATGGTCTTT-CAATTGAGACTTTTCAACAA; SEQ ID NO: 5) primers. (Lower case letters are nonsense sequences which are utilized for effective restriction digestion. Underlined areas are restriction sites which were used for efficient cloning. Bold letters represent the stop codon which was used to engineer a sense nontranslatable construct.) The resulting amplified PCR products were treated with the restriction enzyme, Ncol, and cloned into the plant expression vector pEPT8. Sense or antisense orientation was determined using standard restriction mapping and PCR analysis making use of the positional 35S promoter-and cloned into the plant expression vector pEPT8. Sense or antisense orientation was determined using standard methods. The primer set (P2: agtgctCTCGAGCAATTGAGACTTTTCAACAA; SEQ ID NO: 6) and transgene primers. The expression cassette containing the transgene and plant transcriptional elements, 35S enhancers, 35S promoter, alfalfa mosaic virus RNA4 5’ untranslated sequence and 35S terminator was subsequently cloned into the plant transformation vector pGA482G.

Transformation

[0048] Transformation protocols were modified from those described by Scorza and Cordts, (Plant Cell Rep. 14: 589-592, 1995; Krastanova et al., Plant Cell Rep. 24: 550-554, 1995). Overnight cultures of Agrobacterium strain C58Z707 were grown in LB medium at 28°C in a shaking incubator. Bacteria were centrifuged for five minutes at 5000 rpm (or 3000 rpm) and resuspended in MS liquid medium (OD600 = 0.4-1.0). Callus with globular or heart-shaped embryos was immersed in the bacterial suspension for fifteen to thirty minutes, blotted dry, and transferred to HMG medium with or without acetosyringone (100 uM). The embryogenic calli were cocultivated with the bacteria for forty-eight hours in the dark at 28°C. Next, the plant material was washed in MS liquid plus cefotaxime (300 mg/ml) and carbenicillin (200 mg/ml) two to three times. The material was then transferred to HMG medium containing either 20 or 40 mg/L kanamycin, 300 mg/L cefotaxime, and 200 mg/L carbenicillin to select transgenic embryos. Alternatively, after forty-eight hours of cocultivation with Agrobacterium, embryogenic calli were transferred onto initiation MGE medium containing 25 mg/L kanamycin plus the same antibiotics listed above. All plant material was incubated continuously in the dark at 28°C. After growth on selection medium for three months, embryos were transferred to HMG or MGC without kanamycin for development of hypocotyls. Embryos were then transferred to rooting medium without antibiotics. Nontransformed calli were grown in the same media with and without kanamycin to verify the efficiency of the kanamycin selection and the ability of the plant to regenerate in the presence of the antibiotic.

Analysis of transgenic plants

Isolation of Other Grape Nepovirus-CP Genes

[0050] Any grape nepovirus (for example, GFLLV) isolate can serve as the nucleic acid source for the molecular cloning of a grape nepovirus coat protein (CP) gene. For example, isolation of a GFLLV-CP gene involves the isolation of those DNA sequences which encode a protein exhibiting CP-associated structures, properties, or activities. Based on the GFLLV-CP nucleotide and amino acid sequences described herein (Fig. 1; SEQ ID NOS: 1 and 2), the isolation of additional GFLLV-CP coding sequences is made possible using standard strategies and techniques that are well known in the art.

[0051] In one particular example, the GFLLV-CP sequences described herein may be used, together with conventional screening methods of DNA hybridization screening. Such hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Benton and Davis, Science 196: 180, 1977; Grunstein and Hogness, Proc. Natl. Acad. Sci. USA 72: 3961, 1975; Ausubel et al. ( supra ); Berger and Kimmel ( supra ); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York. In one particular example, all or part of the 'Geneva' isolate nucleotide sequence (described herein) may be used as a probe to screen a recombinant GFLLV DNA library for genes having sequence identity to the coat protein gene of the 'Geneva' isolate. Hybridizing sequences are detected by plaque or colony hybridization according to standard methods, for example those described below.

[0052] Alternatively, using all or a portion of the amino acid sequence of the coat protein gene of the 'Geneva' isolate one may readily design GFLLV-CP-specific oligonucleotide probes, including GFLLV-CP degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either DNA strand and any appropriate portion of the GFLLV-CP sequence (Fig 1; SEQ ID NOS: 1 and 2). General methods for designing and preparing such probes are provided, for example, in Ausubel et al., 1996, Current Protocols in Molecular Biology, Wiley Interscience, New York, and Berger and Kimmel, Guide to Molecular Cloning Techniques, 1987, Academic Press, New York. These oligonucleotides are useful for GFLLV-CP gene isolation, either through their use as probes capable of hybridizing to GFLLV-CP complementary sequences or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies. If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to probe filter replicates from a recombinant DNA library. Recombinant DNA libraries are prepared according to methods well known in the art, for example, as described in Ausubel et al. ( supra ), or they may be obtained from commercial sources.


Construction of Plant Transgenomes

[0054] Most preferably, a grape nepovirus coat protein (for example, a GFLLV-CP) is expressed as a sense translatable mRNA transcript by a stably-transfected grape cell line or by a transgenic grapevine or grapevine component. A number of vectors suitable for either stable or extrachromosomal transfection of plant cells, or for the establishment of transgenic plants are available to the public; such vectors are described in Weissbach and Weissbach (Methods for Plant Molecular Biology, Academic Press, 1989) and Gelvin et al. (Plant Molecular Biology Manual, Kluwer, Academic Publishers, 1990).


[0055] Typically, plant expression vectors include (1) a cloned gene (for example, a nucleic acid molecule which expresses a sense translatable ) under the transcriptional control of 5' and 3' expression control sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive, pathogen- or wound-induced, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

[0056] Once the desired grape nepovirus coat protein nucleic acid molecule is obtained as described above, it may be manipulated in a variety of ways known in the art. For example, a GFLLV-CP DNA sequence of the invention may, if
desired, be combined with other DNA sequences in a variety of ways. The GFLV-CP DNA sequence may be employed with all or part of the gene sequences normally associated with the GFLV-CP. In its component parts, a DNA sequence encoding a GFLV-CP is combined in a DNA construct having a transcription initiation control region capable of promoting transcription in a host grapevine cell.

[0057] In general, the constructs will involve regulatory regions functional in plants which provide for modified production of a GFLV-CP, as discussed herein. For example, the sense nontranslatable sequence for a GFLV-CP or fragment thereof will be joined at its 5’ end to a transcription initiation regulatory region, for example, such as a sequence naturally found in the 5’ upstream region of a plant structural gene. Numerous transcription initiation regions are available which provide for constitutive or inducible regulation.

[0058] For applications where developmental, cell, tissue, hormonal, or environmental expression is desired, appropriate 5’ upstream non-coding regions are obtained from other genes, for example, from genes regulated during meristem development, seed development, embryo development, leaf development, stem development, or tendril development.

[0059] Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the GFLV-CP or any convenient transcription termination region derived from a different gene source (for example, the NOS or 35S CaMV terminators). The transcript termination region will contain preferably at least 1-3 kb of sequence 3’ to the structural gene from which the termination region is derived. Plant expression constructs having GFLV-CP as the DNA sequence of interest for expression (in the sense translatable orientation production of mRNA) may be employed with a wide variety of grapevines. Such genetically-engineered plants are useful for a variety of industrial and agricultural applications. Importantly, this invention is applicable to all grapevines or grapevine components, and will be readily applicable to any new or improved transformation or regeneration methods of grape.

[0060] The expression constructs include at least one promoter operably linked to at least one sense translatable GFLV-CP sequence. An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, for example, Odell et al., Nature 313: 810, 1985). The CaMV promoter is also highly active in monocots (see, for example, Dekeyser et al., Plant Cell 2: 591, 1990; Terada and Shimamoto, Mol. Gen. Genet. 220: 389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see, for example, Kay et al., Science 236: 1299, 1987; Ow et al., Proc. Natl. Acad. Sci., U.S.A. 84: 4870, 1987; and Fang et al., Plant Cell 1: 141, 1989, and McPherson and Kay, U.S. Pat. No. 5,378,142).

[0061] Other useful plant promoters include, without limitation, the nopaline synthase (NOS) promoter (An et al., Plant Physiol. 88: 547, 1988), the octopine synthase promoter (Fromm et al., Plant Cell 1: 977, 1989), the rice actin promoter (Wu and McElroy, WO91/09948), the cyclase promoter (Chappell et al., WO96/36697), and the cassava vein mosaic virus promoter (Verdaguer et al., Plant Mol. Biol. 31: 1129-1139, 1996). Still other exemplary promoters useful in the invention include, without limitation, commelina yellow mottle virus promoter, sugar cane badna virus promoter, rice tungro bacilliform virus promoter, maize streak virus element, and wheat dwarf virus promoter.

[0062] For certain applications, it may be desirable to produce the GFLV-CP sequence in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For this purpose, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to inducible signals such as the environment, hormones, and/or developmental cues. These include, without limitation, genes promoters that are responsible for heat-regulated gene expression (see, for example, Callis et al., Plant Physiol. 88: 965, 1988; Takahashi and Komeda, Mol. Gen. Genet. 219: 365, 1989; and Takahashi et al. Plant J. 2: 751, 1992), light-regulated gene expression (for example, the pea rbcS-3A described by Kuhlemeier et al., Plant Cell 1: 471, 1989; the maize rbcS promoter described by Schäffner and Sheen, Plant Cell 3: 997, 1991; the chlorophyll a/b-binding protein gene found in pea described by Simpson et al., EMBO J. 4: 2723, 1985; the Arabassu promoter; or the rice rbs promoter), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the Em gene of wheat described by Marcotte et al., Plant Cell 1: 969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and Arabidopsis by Straub et al., Plant Cell 6: 617, 1994 and Shen et al., Plant Cell 7: 295, 1995; and wound-induced gene expression (for example, of wund described by Siebertz et al., Plant Cell 1: 961, 1989), organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., EMBO J. 6: 1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al., EMBO J. 7: 1249, 1988; or the French bean β-phaseolin gene described by Bustos et al., Plant Cell 1: 839, 1989), or pathogen-inducible promoters (for example, PR-1, prp-1, or β-1,3 glucanase promoters, the fungal-inducible wiria promoter of wheat, and the nematode-inducible promoters, TobRB7-5A and Hmg-1, of tobacco and parsley, respectively).

[0063] Plant expression vectors may also optionally include RNA processing signals, e.g. introns, which have been shown to be important for efficient RNA synthesis and accumulation (Callis et al., Genes and Dev. 1: 1183, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of
this fact, an intron may be positioned upstream or downstream of a GFLV-CP sequence in the transgene to modulate levels of gene expression.

[0064] In addition to the aforementioned 5’ regulatory control sequences, the expression vectors may also include regulatory control regions which are generally present in the 3’ regions of plant genes (Thornburg et al., Proc. Natl. Acad. Sci. U.S.A. 84: 744, 1987; An et al., Plant Cell 1: 115, 1989). For example, the 3’ terminator region may be included in the expression vector to increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

[0065] The plant expression vector also typically contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the bar gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide BASTA® (Hoechst AG, Frankfurt, Germany).

[0066] In addition, if desired, the plant expression construct may contain a modified or fully-synthetic GFLV-CP sequence which has been changed to enhance the performance of the gene in plants.

[0067] It should be readily apparent to one skilled in the art of molecular biology, especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

Grapevine Transformation

[0068] Upon construction of the plant expression vector, several standard methods are available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (A. tumefaciens or A. rhizogenes) (see, for example, Lichtenstein and Fuller In: Genetic Engineering, vol 6, PWJ Rigby, ed., London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J., In: DNA Cloning, Vol II, D.M. Glover, ed., Oxford, IRI Press, 1985), (2) the particle delivery system (see, for example, Gordon-Kamm et al., Plant Cell 2: 603 (1990); or Sanford et al. U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792.), (3) microinjection protocols (see, for example, Green et al., supra), (4) polyethylene glycol (PEG) procedures (see, for example, Draper et al., Plant Cell Physiol. 23: 451, 1982; or for example, Zhang and Wu, Theor. Appl. Genet. 76: 835, 1988), (5) liposome-mediated DNA uptake (see, for example, Freeman et al., Plant Cell Physiol. 25: 1353, 1984), (6) electroporation protocols (see, for example, Gelvin et al., supra; Dekeyser et al., supra; Fromm et al., Nature 319: 791, 1986; Sheen Plant Cell 2: 1027, 1990; or Jang and Sheen Plant Cell 6: 1665, 1994), and (7) the vortexing method (see, for example, Kindle supra). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. Some exemplary methods for transforming grapes are found in Scorza et al. (Plant Cell Reports 14: 589-592, 1995), Baribault et al. (J. Expt. Bot. 41: 1045-1049, 1990), Mullins et al. (BioTechnology 8: 1041-1045, 1990), Nakano et al. (J. Expt. Bot. 45: 649-656, 1994), Kikkert et al. (Plant Cell Rep. 15: 311-316, 1995), Krastanova et al. (Plant Cell Rep. 1: 550-554, 1995), Scorza et al. (Plant Cell Rep. 14: 589-592, 1994), Scorza et al. (J. Amer. Soc. Hort. Sci. 121: 616-619, 1996), Martinelli et al. (Theor Appl Genet. 88: 621-628, 1994), and Legall et al. (Plant Sci. 102: 161-170, 1994).

As newer methods are available to transform crops or other host cells, they may be directly applied as well.

[0069] The plants for use in the practice of the invention are grapevines (for example, Vitis spp., Vitis spp. hybrids, and all members of the subgenera Euvitis and Muscadinia), including scion or rootstock cultivars. Exemplary scion cultivars include, without limitation, those which are referred to as table or raisin grapes and those used in wine production such as Cabernet Franc, Cabernet Sauvignon, Chardonnay (for example, CH 01, CH 02, CH Dijon), Merlot, Pinot Noir (PN, PN Dijon), Semillon, White Riesling, Lambrusco, Thompson Seedless, Autumn Seedless, Niagara Seedless, and Seval Black. Other scion cultivars which can be used include those referred to as Table or Raisin Grapes, such as Alden, Almeria, Anab-E-Shahi, Autumn Black, Beauty Seedless, Black Corinth, Black Damascus, Black Malvoisie, Black Prince, Blackrose, Bronx Seedless, Burgrave, Calmeria, Campbell Early, Canner, Cardinal, Catawba, Christmas, Concord, Dattier, Delight, Diamond, Dizmar, Duchess, Early Muscat, Emerald Seedless, Emperor, Exotic, Ferdinand de Lesspess, Fiesta, Flame seedless, Flame Tokay, Gasconade, Gold, Himrod, Hunisa, Hussiene, Isabella, Italia, July Muscat, Khandahar, Katta, Kourgane, Kishmishi, Loose Perlette, Malaga, Monukka, Muscat of Alexandria, Muscat Flame, Muscat Hamburg, New York Muscat, Niabell, Niagara, Olivette blanche, Ontario, Pierce, Queen, Red Malaga, Ribier, Rish Baba, Romulus, Ruby Seedless, Schuyler, Seneca, Suavis (IP 365), Thompson seedless, and Thomuscat. They also include those used in wine production, such as Aleatico, Alicante Bouschet, Aligote, Alvalrelhao, Aramon, Baco blanc (22A), Burger, Cabernet franc, Cabernet, Sauvignon, Calzini, Carignane, Charbono, Chardonnay, Chasselas dore, Chenin blanc, Clairette blanche, Early Burgundy, Emerald Riesling, Feher Szagos, Femao Pires, Flora,

[0070] Rootstock cultivars that are useful in the invention include, without limitation, Vitis rupestris Constantia, Vitis rupestris St. George, Vitis california, Vitis girdiana, Vitis rotundifolia, Vitis rotundifolia Carlos, Richter 110 (Vitis berlandieri x rupestris), 101-14 Millarder et de Grasset (Vitis riparia x rupestris), Teleki 5C (Vitis berlandieri x riparia), 3309 Courdenc (Vitis riparia x rupestris), Riparia Gloire de Montpellier (Vitis riparia), 5BB Teleki (selection Kober, Vitis berlandieri x riparia), SO4 (Vitis berlandieri x rupestris), 41B Millardet (Vitis vinifera x berlandieri), and 039-16 (Vitis vinifera x Muscadina). Additional rootstock cultivars which can be used include Couderc 1202, Couderc 1613, Couderc 1616, Couderc 3309, Dog Ridge, Foex 33EM, Freedom, Ganzin 1 (A x R #1), Harmony, Kober 5BB, LN33, Millardet & de Grasset 41B, Millardet & de Grasset 420A, Millardet & de Grasset 101-14, Oppenheim 4 (SO4), Paulsen 775, Paulsen 1045, Paulsen 1103, Richter 99, Richter 110, Riparia Gloire, Ruggeri 225, Saint-George, Salt Creek, Teleki 5A, Vitis rupestris Constantia, Vitis california, and Vitis girdiana.

[0071] In general, transfer and expression of transgenes in plant cells, including grape plants, are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agricultural or commercial interest.

Transgenic Grapevine Regeneration

[0072] Plant cells transformed with a plant expression vector can be regenerated, for example, from single cells, callus tissues, organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, for example, in Vasil supra; Green et al., supra; Weissbach and Weissbach, supra; and Gelvin et al., supra.

[0073] In one particular example, a cloned sense nontranslatable GFLV-CP sequence as a comparative example or a sense translatable construct (for example, a GFLV-CP sequence in the sense orientation having an out-of-reading frame ATG including a stop codon after the initiation codon) under the control of the 35S CaMV promoter and the nopaline synthase terminator and carrying a selectable marker (for example, kanamycin resistance) is transformed into Agrobacterium. Transformation of grapevine with vector-containing Agrobacterium is carried out as described by Scorza and Cordts. Putative transformants are selected after a few weeks on plant tissue culture media containing kanamycin.

[0074] Kanamycin-resistant plant material is then placed on plant tissue culture media without hormones for root initiation.

[0075] Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard detection techniques as described above. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random, and the site of integration can profoundly affect the levels and the tissue and developmental patterns of transgene expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

[0076] Transgenic lines are evaluated for levels of transgene expression. Transformed grapevines that express a sense nontranslatable GFLV-CP sequence having resistance to fanleaf disease relative to control plants are taken as being useful in the invention.

SEQUENCE LISTING

[0076] Cornell Research Foundation, Inc.
NEPOVIRUS RESISTANCE IN GRAPEVINE
07678/023WO2
60/060,384 1997-09-29
FastSEQ for Windows Version 3.0

DNA
Grapevine Fanleaf Virus Coat Protein Gene

CDS
(7) ... (1518)

1

5
gtgagt gga tta gct ggt aga gga gtg att tat atc cct aag gat tgc
Gly Leu Ala Gly Arg Gly Val Ile Tyr Ile Pro Lys Asp Cys
1

5

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Gln Ala Asn Arg Tyr Leu Gly Thr Leu Asn Ile Arg Asp Met Ile Ser
15

20

25

30
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Asp Phe Lys Gly Val Gln Tyr Glu Lys Trp Ile Thr Ala Gly Leu Val
35

40

45

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50

55

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65

70

75

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Arg Ile Thr Ala Ser Ala Asp Pro Val Tyr Thr Leu Ser Val Pro His
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85

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185 190

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Ile Cys Ala Pro Ile Phe Tyr Ser Ile Val Leu Trp Val Val Ser Glu
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Val Tyr Val Glu Gln Asp Gly Ser Phe Glu Val Lys Ile Arg Ser Pro
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Tyr His Arg Thr Pro Ala Arg Leu Leu Ala Asn Glu Ser Glu Arg Asp
290 295 300

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Thr Phe Arg Ile Val Val Arg Leu Pro Ala Asn Ala Phe Thr Gly Leu
50 55 60
Thr Trp Val Met Ser Phe Asp Ala Tyr Asn Arg Ile Ala Ser Arg Ile
65 70 75 80
Thr Ala Ser Ala Asp Pro Val Tyr Thr Leu Ser Val Pro His Trp Leu
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100 105 110
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115 120 125
Arg Leu His Phe Thr Cys Leu Thr Gly Asn Asn Lys Glu Leu Ala Ala
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Asp Trp Gln Ala Val Val Glu Tyr Ala Glu Leu Glu Glu Ala Thr
145 150 155 160
Ser Phe Leu Gly Lys Pro Thr Leu Val Phe Asp Pro Gly Ala Phe Asn
165 170 175
Gly Lys Phe Gln Phe Leu Thr Cys Pro Pro Ile Phe Phe Asp Leu Thr
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Ala Val Thr Ala Leu Arg Ser Thr Gly Leu Thr Leu Gly Gln Val Pro
195 200 205
Met Val Gly Thr Thr Lys Val Tyr Asn Leu Asn Ser Thr Leu Val Ser
210 215 220
Cys Ile Leu Gly Met Gly Met Gly Thr Ile Arg Gly Arg Val His Ile Cys
225 230 235 240
Ala Pro Ile Phe Tyr Ser Ile Val Leu Trp Val Val Ser Glu Trp Asn
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260 265 270
Val Glu Glu Asp Gly Ser Phe Glu Val Lys Ile Arg Ser Pro Tyr His
275 280 285
Arg Thr Pro Ala Arg Leu Leu Ala Asn Gln Ser Gln Arg Asp Met Ser
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Claims

1. A method for selecting and producing a transgenic grapevine or grapevine component having increased resistance to a fanleaf disease in comparison to a non-transgenic grapevine or grapevine component, said method comprising:

   (a) transforming a grape plant cell with a grape nepovirus coat protein nucleic acid molecule or fragment thereof which is capable of being expressed in a sense orientation in said plant cell;

   (b) regenerating a transgenic grapevine or grapevine component from the plant cell; and

   (c) selecting a transgenic grapevine or grapevine component based on its expression of said nucleic acid molecule or fragment thereof wherein said nucleic acid molecule or fragment thereof expresses a grape nepovirus coat protein or fragment thereof and is negative for expression of said grape nepovirus coat protein or fragment thereof as detected by an enzyme-linked immunoassay and wherein said expression increases the resistance of the transgenic grapevine or grapevine component to said fanleaf disease in comparison to a non-transgenic grapevine or grapevine component.

2. The method of claim 1, wherein said nucleic acid molecule or fragment thereof is encoded by a transgene integrated into a transgenic grapevine genome.

3. The method of claim 1, wherein said grapevine is a member of the genus Vitis.

4. The method of claim 1, wherein said grapevine component is a somatic embryo.

5. The method of claim 1, wherein said grapevine component is a scion.

6. The method of claim 1, wherein said grapevine component is a rootstock cultivar.

7. The method of claim 6, wherein said rootstock cultivar is selected from the group consisting of Vitis rupestris St. George, Vitis California, Vitis girdiana, Vitis rotundifolia, Vitis rotundifolia Carlos, Richter 110 (Vitis berlandieri x rupestris), 101-14 Millarder et de Grasset (Vitis riparia x rupestris), Teleki 5C (Vitis berlandieri x riparia), 3309 Courderc (Vitis riparia x rupestris), Riparia Gloire de Montpellier (Vitis riparia), 5BB Teleki (selection Kober, Vitis berlandieri x riparia), S04 (Vitis berlandieri x riparia), 41B Millardet (Vitis vinifera x berlandieri), and 039-16 (Vitis vinifera x Muscadinia).

8. The method of claim 1, wherein said grape nepovirus coat protein nucleic acid molecule is from a grapevine fanleaf virus.

9. The method of claim 1, wherein further comprising a grape nepovirus coat protein nucleic acid molecule or fragment thereof having about 50% or greater sequence identity to SEQ ID NO: 1.

Patentansprüche

1. Verfahren zum Auswählen und Erzeugen eines transgenen Weinstocks oder Weinstockbestandteils, der eine erhöhte Resistenz gegen Reisigkrankheit im Vergleich zu einem nicht-transgenen Weinstock oder Weinstockbestand-
teil aufweist, wobei das besagte Verfahren umfasst:

(a) Transformieren einer Weinpflanzenzelle mit einem Weinneverovirushüllenproteinnukleinsäuremolekül oder einem Fragment davon, das in der besagten Pflanzenzelle in Sinnstrangrichtung exprimiert werden kann;
(b) Neubilden eines transgenen Weinstocks oder Weinstockbestandteils aus der Pflanzenzelle; und
(c) Auswählen eines transgenen Weinstocks oder Weinstockbestandteils aufgrund seiner Expression des besagten Nukleinsäuremoleküls oder Fragments davon, wobei das besagte Nukleinsäuremolekül oder Fragment davon ein Weinneverovirushüllenprotein oder ein Fragment davon exprimiert und wobei mit einem Enzym-gekoppelten Immunspezifitätstest keine Expression des besagten Weinneverovirushüllenproteins oder Fragments davon nachzuweisen ist und wobei die besagte Expression die Resistenz des transgenen Weinstocks oder Weinstockbestandteils gegen die besagte Reisigkrankheit im Vergleich zu einem nicht-transgenen Weinstock oder Weinstockbestandteil erhöht.

2. Verfahren nach Anspruch 1, wobei das besagte Nukleinsäuremolekül oder Fragment davon von einem Transgen kodiert wird, das in ein transgenes Weinstockgenom integriert ist.

3. Verfahren nach Anspruch 1, wobei der Weinstock ein Mitglied des Genus *Vitis* ist.

4. Verfahren nach Anspruch 1, wobei der besagte Weinstockbestandteil ein somatischer Embryo ist.

5. Verfahren nach Anspruch 1, wobei der besagte Weinstockbestandteil ein Ableger ist.

6. Verfahren nach Anspruch 1, wobei der besagte Weinstockbestandteil ein Wurzelstockkultivar ist.

7. Verfahren nach Anspruch 6, wobei der besagte Wurzelstockkultivar aus der Gruppe ausgewählt ist, bestehend aus *Vitis rupestris* St. George, *Vitis californica*, *Vitis girdiana*, *Vitis rotundifolia*, *Vitis riparia* Carlos, Richter 110 (*Vitis berlandieri* x *rupestris*), 101-14 Millardet et de Grasset (*Vitis riparia* x *rupestris*), Teleki 5C (*Vitis berlandieri* x *riparia*), 3309 Courderc (*Vitis riparia* x *rupestris*), *Riparia Glire de Montpellier* (*Vitis riparia*), S5B Teleki (Selektion Kober, *Vitis berlandieri* x *riparia*), S04 (*Vitis berlandieri* x *rupestris*), 41B Millardet (*Vitis vinifera* x *berlandieri*), und 039-16 (*Vitis vinifera* x *Muscadinia*).

8. Verfahren nach Anspruch 1, wobei das besagte Weinneverovirushüllenproteinnukleinsäuremolekül von einem Reisigvirus stammt.

9. Verfahren nach Anspruch 1, wobei es des Weiteren ein Weinneverovirushüllenproteinnukleinsäuremolekül oder Fragment davon umfasst, das eine ca. 50% oder größere Sequenzidentität zu SEQ ID NO: 1 aufweist.

**Revendications**

1. Procédé pour la sélection et la production d’une vigne ou d’un composant de vigne transgénique ayant une résistance accrue à la maladie de la feuille en éventail par comparaison avec une vigne ou un composant de vigne non-transgénique, le dit procédé comprenant:

(a) la transformation d’une cellule de plant de vigne avec une molécule ou un fragment de molécule d’acide nucléique de protéine de revêtement du népovirus du raisin, qui est capable d’être exprimée dans une orientation sens dans ladite cellule végétale;
(b) la régénération d’une vigne ou d’un composant de vigne transgénique à partir de la cellule végétale; et
(c) la sélection d’une vigne ou d’un composant de vigne transgénique sur la base de son expression de ladite molécule d’acide nucléique ou d’un fragment de celle-ci, tandis que ladite molécule d’acide nucléique ou ledit fragment de celle-ci exprime une protéine de revêtement du népovirus du raisin ou un fragment de celle-ci et est négative quant à l’expression de ladite protéine de revêtement du népovirus du raisin ou d’un fragment de celle-ci, comme cela est détecté par un test immuno-enzymatique et tandis que ladite expression augmente la résistance de la vigne ou du composant de vigne transgénique vis-à-vis de ladite maladie des feuilles en éventail par comparaison avec une vigne ou un composant de vigne non-transgénique.

2. Procédé selon la revendication 1, dans lequel ladite molécule d’acide nucléique ou ledit fragment de celle-ci est codé par un transgène intégré dans un génome de vigne transgénique.
3. Procédé selon la revendication 1, dans lequel ladite vigne est un membre du genre Vitis.

4. Procédé selon la revendication 1, dans lequel ledit composant de vigne est un embryon somatique.

5. Procédé selon la revendication 1, dans lequel ledit composant de vigne est un scion.

6. Procédé selon la revendication 1, dans lequel ledit composant de vigne est un rhizome.

7. Procédé selon la revendication 6, dans lequel ledit cultivar de rhizome est choisi dans le groupe consistant en Vitis rupestris St. George, Vitis california, Vitis girdiana, Vitis rotundifolia, Vitis rotundifolia Carlos, Richter 110 (Vitis berlandieri x rupestris), 101-14 Millarder et de Grasset (Vitis riparia x rupestris), Teleki 5C (Vitis berlandieri x riparia), 3309 Courdrc (Vitis riparia x rupestris), Riparia Gloire de Montpellier (Vitis riparia), 5BB Teleki (sélection Kober, Vitis berlandieri x riparia), S04 (Vitis berlandieri x rupestris), 41B Millardet (Vitis vinifera x berlandieri), et 039-16 (Vitis vinifera x Muscadinia).

8. Procédé selon la revendication 1, dans lequel ladite molécule d’acide nucléique de protéine de revêtement du népovirus du raisin est issue d’un virus de la feuille en éventail de la vigne.

9. Procédé selon la revendication 1, comprenant en outre une molécule d’acide nucléique de protéine de revêtement du népovirus du raisin ou un fragment de celle-ci, ayant environ 50% ou plus d’identité de séquence vis-à-vis de SEQ ID NO: 1.
FIG. IA
Gene constructs used to transform grape

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FIG. 2
## Table

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## Figure 3

![Graph showing changes in A405 nm over time for different conditions](image-url)
REFERENCES CITED IN THE DESCRIPTION

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