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Stable insertion and expression of the movement protein gene of Grapevine Virus A (GVA) in grape (*Vitis rupestris* S.)

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Summary

Transformation with the movement protein gene of Grapevine virus A, both in sense and antisense orientation, was done in *Vitis rupestris* S. somatic embryos through LBA 4404 *Agrobacterium tumefaciens* co-cultures, and plantlets were regenerated. Molecular assays of regenerated plantlets, after 4 years of micropropagation cycles, verified stable insertion and expression of the foreign genes in both orientations. Plants expressing the sense form of the viral gene, exhibited morphological and physiological anomalies, such as slow growth, suppression of buds and flowering and tendril development.

K e y w o r d s : Grape; Grapevine virus A (GVA); movement protein; pathogen-derived resistance; transgenic grapevine.

Introduction

Among the genes related to agronomically important traits which have been transferred to grapes, genes associated with virus resistance are most common (Kikkert *et al.* 2001; Martinelli and Mandolino 2001). The expression of viral protein genes in plants coding for structural and non-structural proteins - a strategy already known as pathogen-derived resistance (PDR) (Hamilton 1980) - proved effective in several crops to induce protection against virus infections (Sanford and Johnston 1985; Martelli *et al.* 1999); moreover, this technique offers interesting perspectives to investigate virus-plant interactions.

In grapevine, mainly coat protein genes of several viruses were inserted (Le Gall *et al.* 1994; Gölles *et al.* 2000; Krastanova *et al.* 2000; Mauro *et al.* 2000), and the first field trial of transgenic grapes concerned a rootstock (SO 4) expressing the coat protein of Grapevine fanleaf virus (GFLV) (notification number B/FR/94/11/04). However, functional genes, such as movement protein and replicase genes, were less adopted for exploiting pathogen-derived resistance in grape (Martinelli *et al.* 2000).

Viral infections which can substantially reduce grapevine yields may be overcome by sanitary selection and certification. This is particularly important if virus is transmitted through insects, and healthy stocks are re-infected; this is the case of the rugose wood complex, an ubiquitous disease of grapevine (Martelli 1993), in the aetiology of which several viruses transmitted by pseudococcid mealybugs are involved (La Notte *et al.* 1997). Within the 4 syndromes of the rugose wood complex, Kober stem grooving (KSG) was consistently associated with Grapevine virus A (GVA) (Boscia *et al.* 1997), a single stranded RNA virus of 800 nm length and 15 nm thickness with a genome length of 7.4 kb (Minafra *et al.* 1994, 1997). To obtain the resistance to GVA with molecular strategies, we tested the potential of the movement protein gene, as this protein is the key factor of the "slow cell to cell movement" (Carrington *et al.* 1996) of the viruses through plant tissues.

The strategy applied in a previous study (Martinelli *et al.* 2000) gave promising results since an interesting level of protection was found in tobacco plantlets expressing the GVA movement protein gene in both sense and antisense orientations, when challenge-inoculated with the homologous virus. A higher degree of tolerance to virus infection was detected when the antisense construct was expressed; moreover, a partial remission of symptoms and a decrease of virus accumulation was observed in the apical leaves grown in the third week after inoculation, which was never detected when the GVA coat protein gene was used for transformation (Minafra *et al.* 1998).

In addition to the attempt to produce resistant plants, the expression of this functional gene in grape tissues provided us with an interesting tool for studying viral diffusion in the plant. The movement protein gene of GVA, both in sense and antisense orientations, was successfully transferred to *V. rupestris* somatic embryos, and plants were regenerated (Martinelli *et al.* 2000). Stability and expression of both orientations of this viral gene were assayed in transgenic plants after 4 years of micropropagation cycles and results are presented here.

Material and Methods

C o n s t r u c t p r e p a r a t i o n : The GVA movement protein gene was amplified from a cDNA template synthesized on purified viral RNA using the following specific primers: Amp1 upstream, 5'-AAGGTG AGGATC CCCATG GCGCAAG-3'; Amp2 downstream, 5'-AGA AGGATCC TCAGTTGGTTGG-3'. The amplified fragment of 850 bp was directionally ligated, after digestion and gel elution, in the sites NcoI and BamHI of a pRT103 transcriptional cassette

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preserving the natural ATG and stop codon, under the CaMV 35S promoter. To allow the transcription of an antisense RNA, the cloned fragment was excised with the same enzymes and religated, after blunt-ending, in the SmaI site of the same vector, selecting the opposite orientation. The cassettes containing the promoter and the sense or antisense constructs were inserted in the HindIII site of pGA482 (Pharmacia Biotech, Uppsala, Sweden), beside the NPT II selection gene, which was transformed in *A. tumefaciens* LBA4404 by triparental mating (MARTINELLI *et al.* 2000).

Gene transfer, transgenic plant regeneration and establishment of subclones: The GVA movement protein gene, either in sense or antisense orientation, was transferred via *Agrobacterium* to *Vitis rupestris* S. somatic embryos induced to secondary embryogenesis, and plantlets were regenerated according to an already established protocol available in our laboratory (MARTINELLI and MANDOLINO 1994, 2001). Several plants from different lines, after rooting *in vitro*, were acclimated in soil according to IACONO and MARTINELLI (1998) and have been successfully grown in the greenhouse. Besides, subclones were established starting from respectively 3 and 2 plant lines resulted transgenic for the sense and antisense orientation of the GVA movement protein genes: monthly nodal micropropagations were done for 4 years on NN-based medium (NITSCH and NITSCH 1969) with 15 $g\,l^{-1}$ sucrose and 9 $g\,l^{-1}$ agarose, and incubated at 25 °C with a 16-h photoperiod (70 $\mu mol\ m^{-2}\ s^{-1}$ cool white light), according to MARTINELLI *et al.* (1993).

Molecular analysis of transgenic grape: Genomic DNA was extracted from 300 mg of young leaves of micropropagated V. rupestris S., either from control and transformed plants (both in sense and antisense orientation) with the CTAB method (ROGERS and BENDICH 1985), and exogenous gene presence was detected by Polymerase Chain Reaction (PCR); the upstream Amp1 and the downstream Amp2 primers were used for amplifying the 850 bp sequence of the GVA movement protein gene, by the Platinum Taq DNA Polymerase (Invitrogen, San Diego, CA, USA). A 14 min step at 94 °C was followed by 30 amplification cycles (respectively 1.5 min denaturation at 94 °C, 1 min annealing at 56 °C, 1 min extension at 72 °C) and a 10 min final elongation step at 72 °C. PCR products were separated by agarose gel electrophoresis according to SAMBROOK et al. (1989).

In addition, Southern blot analysis was performed for verifying exogenous gene insertion, according to SAMBROOK *et al.* (1989); genomic DNA was digested by Xho I (restriction site of which is contained in the pRT103 polylinker before of the inserted gene), separated by agarose gel electrophoresis and transferred by capillarity on nylon Hybond N+ (Amersham Biosciences) filter. Hybridizations were carried out at high stringency conditions according to Gene Images kit instructions (Amersham Biosciences) with a fluorescein-labelled, PCR-generated DNA fragment (Random prime labelling kit, Amersham Biosciences, Little Chalfont, UK) corresponding to the GVA movement protein gene. The membrane filter was exposed to a radiographic film (Kodak X-Omat).

For the transcription assays of the inserted gene, total RNA was extracted according to RENAULT *et al.* (2000), from 300 mg of leaves of both control and transformed sense and antisense plants after a 4-year micropropagation. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed in the presence of the previously described upstream Amp1 and downstream Amp2 primers, the RNase OUT Recombinant Ribonuclease Inhibitor and M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA). Similar conditions were used for PCR and RT-PCR amplification, however, an additional initial step of 60 min at 42 °C was accomplished for the reverse transcription. Resulting fragments were separated on agarose gel electrophoresis according to SAMBROOK *et al.* (1989). For a better resolution, hybridization was performed with the same non-radioactive probe, as described above for the Southern blot analysis.

Results and Discussion

Considering the adoption of molecular strategies for breeding programs, the stable insertion as well as the permanent expression of a foreign gene in a transgenic plant is a crucial aspect of a genetic transformation strategy. These are essential prerequisites to be checked before performing any further evaluation of the new inserted trait.

In the present research, within many independent genetic transformation trials, as confirmed with molecular assays, several transgenic lines were regenerated; among these, 3 and 2 lines were selected, being transgenic for the sense and antisense form of the GVA movement protein genes respectively, and during a 4-year micropropagation, numerous subclones of each line were obtained.

The effectiveness of our transformation technique for producing stable transformed plants was already proven for *V. rupestris* by inserting marker genes, as previously reported (MARTINELLI and MANDOLINO 1996, 2001); similar performance was also obtained with the viral gene. Indeed, PCR assays proved the presence of foreign genes in both orientations in the extracted plant DNA (Fig. 1), and Southern blot analyses confirmed the insertion of the viral genes in the plant genomes (Fig. 2). For this latter test, XhoI was chosen as the most suitable restriction enzyme, according to the construct restriction map and the results of previous Southern blot assays , where EcoRI and HindIII were adopted (MARTINELLI *et al.* 2000). DNA length of the detected fragments (25 kb, 18 kb and 5kb) resulted in a larger than ex-

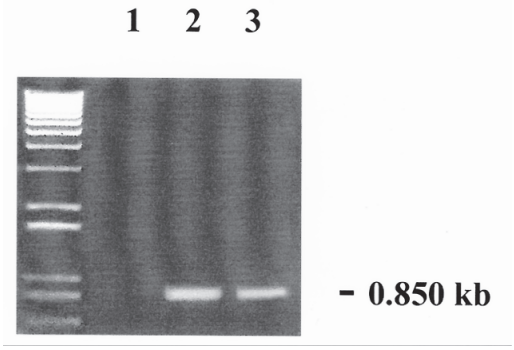

Fig. 1: PCR analysis on genomic DNA extracted from *V. rupestris*: Untransformed control (lane 1), transformed with the sense (lane 2) and antisense (lane 3) orientations of the GVA movement protein gene.

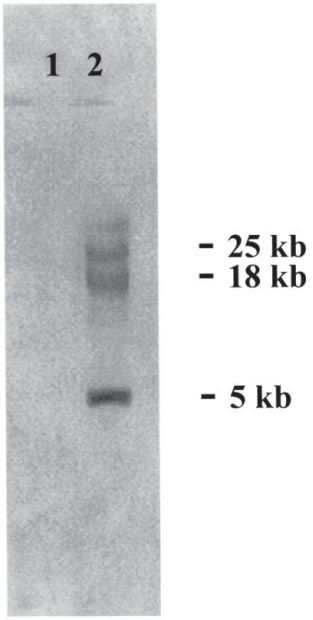

Fig. 2: Southern blot analysis of genomic DNA of *V. rupestris* untransformed control (lane 1) and transgenic (lane 2) respectively, after digestion with XhoI and hybridization with the fluorescein-labelled GVA movement protein gene. Here a sample transformed with the antisense orientation of the gene is reported. Molecular size of the hybridizing bands is compared with a co-migrated λ DNA-HindIII marker.

pected target sequence (*Agrobacterium tumefaciens* T-DNA region with Grapevine virus A (GVA) movement protein), proving the insertion of recombinant DNA in the plant genome. The presence of several fragments suggests that foreign gene insertion occurred in more than one region of the plant genome.

Finally, the transcription of the foreign viral gene was also proven in the subclones after a long period of micropropagation cycles since RT-PCR analysis detected the corresponding gene transcript of 850 nt (Fig. 3).

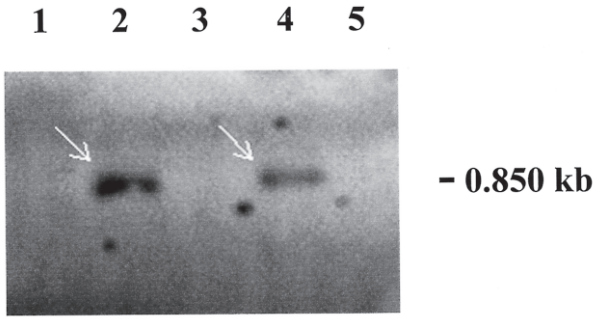

Fig. 3: Southern blot analysis of the RT-PCR products amplified from *V. rupestris* extracts, hybridized with fluorescein-labelled GVA movement protein gene; untransformed control (1); two randomly-choosen transgenic plants transcribing the antisense orientation of the gene (2 and 4) after a 4-year-micropropagation, and PCR conrols without reverse transcription (3 and 5).

It is worth noting that anomalies concerning bud ontogenesis were observed in the transgenic plants. These abnormalities - which resulted in a developmental arrest of both apical and axillary buds and the development of tendrils and flowers instead (Fig. 4) - were dramatically evident

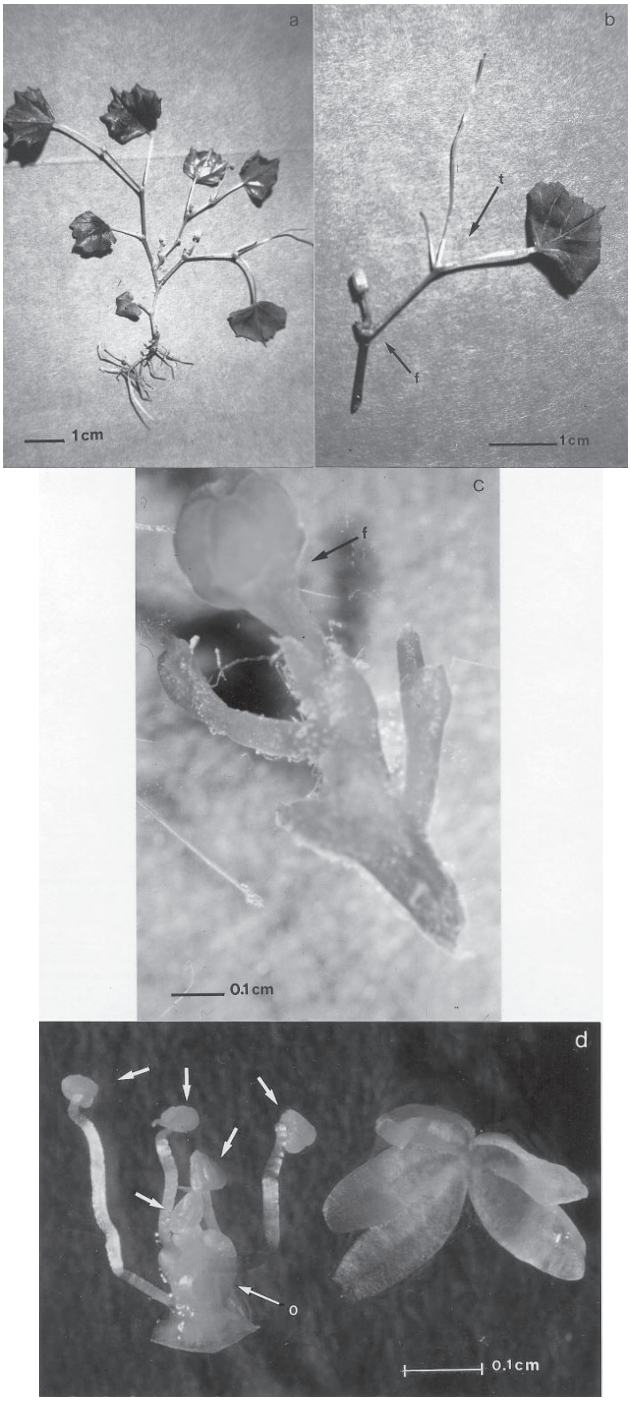

Fig. 4: Arrest of bud, flower and tendril development of bud meristems in *V. rupestris* plantlets transcribing the Grapevine virus A (GVA) movement protein gene in the sense orientation, in a whole plant (**a**) and in an internode (**b**; t = tendril, f = flower). The flower, closed (**c**; f = flower) and dissected (**d**), shows the typical structure of the species, with a 5-petal calyptra, one rudimental ovary (d: o) and 5 anthers (d: arrows).

in plants transcribing the sense form of the movement protein gene of Grapevine virus A (GVA); in these plants, the progressive disappearance of buds (Fig. 4 a and b) resulted in a loss of propagules for further micropropagations and, thus, almost total plant loss. Flowers had 5 anthers and one rudimental ovary, according to the typical *V. rupestris* morphology (Fig. 4 c and d). Often, a slow growth of the plants

during the micropropagation cycles was noticed in the samples exhibiting the described bud anomaly.

The occurrence of mutations (somaclonal variation) associated with the long *in vitro* culture period of the transgenic plants could be advocated as a possible explanation of the bud development inhibition and alternative organ differentiation. A quite similar abnormal plant development was described, for instance, in *Arabidopsis thaliana* expressing mutated forms of a gene (REVOLUTA) involved in bud meristem growth (TALBERT *et al.* 1995). In our grapes, however, such an abnormal status was rarely observed when the antisense form of the viral gene was expressed (Fig. 5) and was never found in control plants. Moreover, this altered phenotype was never observed in marker gene expressing plants (MARTINELLI *et al.* 1993; MARTINELLI and MANDOLINO 1994) produced with the same protocol and kept *in vitro* under the same culture conditions, even after almost 10 years of micropropagation cycles. Thus, we believe that such phenotypical development would be more likely related to the interference between the viral gene, when constitutively expressed in the sense form, and the pathway leading to the meristem development of the plants, rather than to somaclonal variation.

Fig. 5: Rooted *V. rupestris* plantlet expressing the antisense form of the movement protein gene of Grapevine virus A (GVA) after one month nodal micropropagation; development of shoots from internods is clearly visible.

These physiological plant anomalies, as well as the already mentioned encouraging results obtained in transgenic tobacco plants (MARTINELLI *et al.* 2000) led us to consider the antisense form of the GVA movement protein gene as a promising tool for exploiting the pathogen-derived resistance strategy in grape. Moreover, while the sense form of movement protein transgenes are reported to enhance viral movement in transgenic plants or even assist the movement of defective viruses from other groups, several studies, at the opposite, reported a significant resistance from expression of antisense RNAs. Thus, antisense expression resulted in a reduction of the potential concern of epidemiological risks (HAMMOND *et al.* 1999). Furthermore, in the light of an eventual *in vivo* release of the plants, and with regard to a reduction of environmental contamination, the strategy based on antisense RNA appears to be very convenient since no transgenic proteins are translated and thus released.

The stability of the viral movement protein gene in the transgenic plant genomes allowed us to design experiments for assessing plant protection from viral infection and for studying the diffusion of the viruses through the plant tissues. For these assays, *in vitro* grafting of transgenic shoots on GVA-infected rootstocks are already established.

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