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DEVELOPMENT OF GENETIC VECTOR CONSTRUCTS AGAINST ALFALFA MOSAIC VIRUS (AMV) INFECTING ALFALFA USING RNAI AND VIGS TECHNOLOGIES

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Abstract
Alfalfa (Medicago sativa L.) is the principal perennial forage legume of Uzbekistan and an important component of crop rotation systems because of its high protein content and its ability to fix atmospheric nitrogen. Alfalfa mosaic virus (AMV), a member of the genus Alfamovirus (family Bromoviridae), is one of the most widespread pathogens of this crop and causes mosaic symptoms, stunting, and substantial losses of green mass and seed yield. Because conventional control measures are poorly effective against a virus transmitted non-persistently by aphids and through seed, RNA-based approaches have attracted growing attention. This article outlines a strategy for designing genetic vector constructs directed against AMV by means of RNA interference (RNAi) and virus-induced gene silencing (VIGS). It describes the selection of target regions in the viral genome, the design of hairpin RNA (hpRNA) expression cassettes and VIGS vectors, the delivery and verification procedures, and the criteria for evaluating resistance.

Keywords: Alfalfa, Alfalfa mosaic virus, RNA interference, virus-induced gene silencing, coat protein gene, hairpin construct, vector design.

Introduction

Forage production depends strongly on perennial legumes, and alfalfa occupies a leading position among them because it provides several cuttings per season, improves soil structure, and enriches the soil with biologically fixed nitrogen. In irrigated regions of Uzbekistan, alfalfa is also valued as a crop that supports soil fertility restoration in cotton and wheat rotations. The long productive life of an alfalfa stand, however, favors the accumulation of viral pathogens. Once a plant is infected, the virus persists in the crop for several years and cannot be eliminated by agronomic practices or chemical treatments.

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Alfalfa mosaic virus is among the most economically significant viruses of alfalfa and has an exceptionally wide host range that includes many legumes, vegetables, and weeds. The virus is transmitted by more than twenty aphid species, including the pea aphid and the green peach aphid, in a non-persistent manner, which means that acquisition and transmission take place within seconds or minutes of probing. Insecticides therefore provide little protection, since the virus can be transmitted before an aphid is killed. In addition, AMV is transmitted through seed, which allows it to spread over long distances with planting material. The infected plants show yellow or light green mosaic patterns on leaves, shortened internodes, reduced vigor, and a decline in forage quality and seed production.

Breeding for resistance to AMV in alfalfa is complicated by the tetraploid and outcrossing nature of cultivated alfalfa and by the scarcity of strong natural resistance genes. Against this background, the sequence-specific defense mechanism known as RNA silencing offers a promising alternative. This mechanism is a natural antiviral defense in plants: double-stranded RNA produced during viral replication is processed by Dicer-like (DCL) enzymes into small interfering RNAs (siRNAs) of 21 to 24 nucleotides, which are loaded onto Argonaute (AGO) proteins and guide the cleavage of homologous viral RNA. Two biotechnological tools based on this mechanism are of particular interest for virus control. The first is RNAi, in which a transgene is designed to produce double-stranded RNA homologous to the viral genome and thus to prime the plant for efficient silencing of the invading virus. The second is VIGS, in which a modified viral vector carrying a fragment of a target sequence is used to trigger silencing, and which is widely employed for rapid functional analysis of genes and for testing silencing triggers before stable transformation is attempted.

The aim of this work is to outline the principles for creating genetic vector constructs against AMV that combine RNAi and VIGS technologies, and to describe a logical sequence of steps from target selection to evaluation of antiviral activity in alfalfa

The design of any silencing construct begins with knowledge of the viral genome. The AMV genome is divided into three positive-sense single-stranded RNA molecules, designated RNA 1, RNA 2, and RNA 3, which are packaged in separate bacilliform particles. RNA 1 encodes the P1 protein, which contains methyltransferase and helicase domains and participates in viral replication. RNA 2 encodes the P2 protein, which carries the RNA-dependent RNA polymerase motifs. RNA 3 is dicistronic and encodes the movement protein (MP), required for cell-to-cell spread, and the coat protein (CP). The coat protein is translated from a subgenomic RNA 4 that is synthesized from the minus strand of RNA 3.

A distinctive feature of alfamoviruses is that the coat protein is needed to initiate infection: the genomic RNAs are not infectious alone, and activation requires the binding of a few coat protein molecules to the 3′ untranslated region. Moreover, the coat protein is abundantly produced, which makes the CP gene and its subgenomic RNA attractive silencing targets. Another practical consideration is the variability among strains. Isolates of AMV differ in sequence and are grouped into subgroups, so a silencing trigger should be directed toward regions conserved across local isolates. For this reason, the collection of infected alfalfa leaf samples from several fields, total RNA extraction, and reverse transcription followed by sequencing of the CP and MP regions is considered the first practical step. Sequences obtained from local isolates should be aligned with those deposited in public databases in order to detect the most conserved segments.

For RNAi-mediated resistance, the most efficient design is an intron-spliced hairpin RNA cassette.

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In this design, a fragment of the viral sequence is inserted twice in inverted orientation, separated by a functional intron, and placed under the control of a strong promoter. After transcription, the two arms of the transcript base-pair after intron removal and yield a long double-stranded stem, which is an excellent substrate for DCL enzymes. Experience with plant viruses has shown that intron-spliced hairpins induce silencing far more efficiently than sense-only or antisense-only constructs.

Several parameters influence the quality of the construct and should be considered during the design.

Target region. A fragment of 200 to 500 base pairs from the coat protein gene, or from a fusion of conserved CP and MP segments, is generally sufficient to generate a diverse pool of siRNAs. Fragments with high identity (preferably above 90 percent) among the sequenced local isolates are selected so that the construct is effective against several strains.

Specificity. The candidate fragment is checked against the alfalfa and Medicago truncatula transcriptomes with bioinformatic tools for siRNA prediction, in order to avoid stretches of 21 nucleotides or more that could silence host genes. Fragments with a moderate GC content and without long homopolymer runs are preferred.

Promoter and terminator. The cauliflower mosaic virus 35S promoter is widely used for constitutive expression in legumes. If tissue specificity is required, vascular or phloem-specific promoters can be considered, since AMV moves through the phloem and is acquired by aphids from epidermal and mesophyll cells. The octopine synthase or nopaline synthase terminator is commonly placed after the cassette.

Cloning system. The cassette can be assembled with standard restriction cloning in vectors of the pHANNIBAL and pKANNIBAL series and then transferred into a binary vector such as pART27 or pCAMBIA. Alternatively, Gateway-compatible vectors of the pHELLSGATE type allow recombination of a single PCR product into both arms in one step, which reduces the number of cloning steps and the risk of recombination in bacteria.

A plant selection marker, such as the neomycin phosphotransferase II gene or the hygromycin phosphotransferase gene, is included in the T-DNA region to permit selection of transformed tissue.

VIGS vectors are derived from viral genomes that are modified to carry a short insert of the target sequence. When the recombinant virus replicates in the plant, it produces double-stranded replication intermediates that include the insert, and the plant silencing machinery processes them into siRNAs that act against any homologous RNA, including the insert-matching sequence of the challenging virus. In legumes, vectors based on Tobacco rattle virus (TRV), Bean pod mottle virus (BPMV), and Pea early browning virus (PEBV) have been employed. The TRV system is particularly convenient, as it consists of two plasmids, pTRV1 and pTRV2, which are delivered by Agrobacterium tumefaciens and are able to infect a broad range of hosts, including Medicago species.

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For the creation of a VIGS construct against AMV, a 150 to 300 bp fragment of the selected viral region is amplified by PCR with primers carrying appropriate restriction sites or recombination arms and is inserted into the multiple cloning site of pTRV2. The recombinant plasmid is introduced into Agrobacterium, and a mixture of cultures carrying pTRV1 and the recombinant pTRV2 is delivered into young alfalfa seedlings by vacuum infiltration, syringe infiltration, or cotyledon-node injection. An empty pTRV2 vector and a vector carrying a reporter gene, for example phytoene desaturase, are used as controls: the latter induces photobleaching and thus visually confirms that the silencing machinery is operating in the experimental conditions.

The main role of VIGS in this strategy is that of a rapid screening platform. Transient assays can be completed in a few weeks, whereas the creation of stable transgenic alfalfa lines takes several months. The efficiency of different viral fragments, fragment lengths, and combinations of targets can therefore be compared quickly, and only the most effective sequences are transferred to the stable RNAi cassette. Plants pre-silenced with the VIGS construct are challenged by mechanical inoculation with AMV or by feeding of viruliferous aphids, and the accumulation of the virus is compared with that in control plants.

Stable transformation of alfalfa is performed mainly by Agrobacterium-mediated gene transfer into explants such as leaf discs, petioles, or cotyledons, followed by somatic embryogenesis. Because regeneration capacity depends strongly on the genotype, highly regenerable clones or cultivars are generally chosen for transformation. Explants are co-cultivated with Agrobacterium tumefaciens strains such as EHA105 or LBA4404 that harbor the binary vector, transferred to selection medium, and regenerated through callus and embryo stages into complete plantlets, which are then acclimatized in greenhouse conditions.

The molecular verification of putative transformants includes several steps. First, PCR with primers specific to the promoter, the intron, and the marker gene confirms the presence of the T-DNA. Second, Southern blot or digital PCR estimates the copy number of the insertion, because single-copy insertions typically give more stable expression. Third, the production of transgene-derived siRNAs is detected by small-RNA northern blotting or by stem-loop RT-PCR. Finally, the expression of the hairpin transcript is quantified by RT-qPCR.

The quality of the generated plant material is judged by the response to virus challenge. Transgenic and control plants are inoculated with a verified AMV isolate under insect-proof conditions. The symptoms are scored on a numerical scale during several weeks, the viral titer in upper non-inoculated leaves is measured by double-antibody sandwich ELISA and RT-qPCR, and the proportion of infected plants is calculated for every line. Lines that show no symptoms and a low or undetectable virus titer are considered resistant, and their progeny is analyzed to confirm the stable inheritance of the trait.

The combination of VIGS and RNAi has several advantages for alfalfa. First, it makes the development process more efficient, because the most active silencing triggers are identified in a short time before time-consuming transformation is initiated. Second, resistance based on RNA silencing is sequence-specific and does not require the accumulation of foreign proteins, which can be an advantage from the point of view of biosafety assessment. Third, the construct can be extended by joining fragments from different viral genes or from different viruses into a single cassette, creating the possibility of multiple virus resistance. This is important because alfalfa is frequently co-infected with several viruses.

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There are also limitations that should be recognized. Viruses may overcome RNA silencing through suppressor proteins or through the accumulation of point mutations in the targeted region, and a trigger that depends on a single short fragment can be broken by highly variable strains. The use of conserved regions, the combination of several targets, and the periodic monitoring of local virus populations reduce this risk. In addition, the tetraploid and heterozygous genome of alfalfa creates variability in transgene expression among regenerants, so that large numbers of independent lines have to be analyzed. The environment also influences silencing efficiency: low temperatures generally reduce the accumulation of siRNAs, and the performance of lines should therefore be checked under different temperature regimes. Finally, the use of transgenic plants in agriculture is regulated, and the practical application of such constructs requires compliance with national biosafety legislation and risk assessment. For this reason, the VIGS component and non-transgenic delivery approaches, such as the topical application of double-stranded RNA to leaves, may be valuable for the development of less regulated protection strategies.

Alfalfa mosaic virus remains a serious threat to alfalfa production because it is spread by aphids and seeds and cannot be controlled with chemical means. RNA silencing technologies provide a precise and flexible route for engineering resistance. The proposed approach begins with sequencing local AMV isolates and selecting conserved target fragments of the coat protein and movement protein genes, continues with the construction of pTRV2-based VIGS vectors for rapid testing, and ends with the preparation of intron-spliced hairpin RNAi cassettes in binary vectors for stable transformation of alfalfa. Molecular verification by PCR, RT-qPCR, and small-RNA detection, together with virus challenge tests, provides the objective basis for selecting resistant lines. The implementation of this scheme can contribute to the development of virus-resistant alfalfa genotypes and to the sustainable production of forage in Uzbekistan.

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