A study of African cassava mosaic virus gene expression for the development of virus resistance

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A study of African Cassava Mosaic Virus
gene expression for the development of
virus resistance

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Doctor of Natural Sciences

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to Reto
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Zusammenfassung


Aufgrund dieser Resultate wurde der ORF der Barnase, einer unspezifischen RNAse von *Bacillus amyloliquefaciens*, so kloniert, dass er vom AV-Promotor kontrolliert wird; der ORF der Barstar, des spezifischen Inhibitors der Barnase, wird vom AC-Promotor kontrolliert. Die einzigartige Regulation des ACMV-Promotors, welche ausschliesslich vom Virus selbst induziert werden kann, und ein fein abgestimmtes Toxin-/Anti-Toxin-System sollten es möglich machen, eine hypersensitive Reaktion zu simulieren. Da die Regeneration von transgenen Pflanzen, welche eine

Als letzter Schritt wurde versucht, bereits publizierte Transformations- und Regenerations-Protokolle an eine afrikanische Manioksorte anzupassen, um in naher Zukunft neue ACMV-resistente Manioksorten zu produzieren.
Summary

Cassava (*Manihot esculenta* Crantz) is a perennial shrub, belonging to the family *Euphorbiaceae*. It is one of the most important sources of calories produced within the tropics and provides food for over 500 million people. Also in Africa one of the main staples is cassava, however, its production is now seriously threatened by the African cassava mosaic disease. It is caused by African cassava mosaic virus (ACMV), a geminivirus of the *Begomovirus* group, which comprises viruses having a single or bipartite genome (DNA A and DNA B) transmitted by whitefly. Until now there is no completely ACMV-resistant cassava variety available, furthermore, there is no method known to protect the plants in the field from the virus, except by trying to control the spread of the vector using agrochemicals. Since traditional breeding of cassava is constrained, genetic engineering could complement existing breeding programs. Using genetic engineering, the feasibility of a novel virus resistance strategy based on the regulated ACMV promoter was assessed in this thesis.

As a prerequisite for this, ACMV promoter regulation was studied with two different luciferase genes under the control of the bidirectional ACMV promoter. Using viral DNA A for transient transformation experiments to mimic a viral infection, it could be shown that the coat protein promoter (AV promoter) is up-regulated (around 50-fold), while the promoter of the replication associated protein (AC promoter) is down-regulated (2-fold) by DNA A in both tobacco and cassava protoplasts. The regulation of the DNA B promoter is different, as both v- and c-sense expression are enhanced by the DNA A in tobacco protoplasts, while in cassava protoplasts the expression in c-sense was enhanced more than in v-sense. Analogous experiments were performed in transgenic tobacco plants, containing the ACMV-promotor-luciferase constructs. Transient results could be confirmed, but the up-regulation of the AV promoter was less pronounced.

Based on these results, the barnase ORF, producing an unspecific ribonuclease of *Bacillus amyloliquefaciens*, was cloned under the control of the AV promoter and the barstar ORF, producing a specific inhibitor of the barnase, was cloned under the control of the AC promoter. The unique properties of the regulation of the ACMV promoter and its specificity for the virus should make it possible to mimic a hypersensitive reaction by using this finely tuned toxin/anti-toxin system. As regeneration of plants containing complete copies of this construct proved difficult, a sORF was inserted downstream of the barnase to reduce its translation. Transgenic plants were tested in a viral replication assay using particle bombardment; for all analyzed plants reduced viral replication (1 – 30% of the replication in wild type plants) could be shown.
As a last step the transferability and adaptability of published transformation and regeneration protocols to an African cassava cultivar were assessed in order to allow the engineering of African cassava mosaic virus resistant cassava in the near future.
**Abbreviations**

- 2,4-D: 2,4-dichlorophenoxy-acetic acid
- AC promoter: DNA A promoter in c-sense
- ACMD: African cassava mosaic disease
- ACMV: African cassava mosaic virus
- ATP: adenosine triphosphate
- AV promoter: DNA A promoter in v-sense
- BA: 6-Benzylaminopurine
- BC promoter: DNA B promoter in c-sense
- BCTV: beet curly top virus
- BMS: basic MS medium
- bp: basepair
- BV promoter: DNA B promoter in v-sense
- CaMV: cauliflower mosaic virus
- CBN: Cassava Biotechnology Network
- CIAT: International Center for Tropical Agriculture, Columbia
- CP: coat protein
- DI DNA: defective interfering DNA
- DIG: digoxigenin
- DNA: deoxyribonucleic acid
- ds DNA: double-stranded DNA
- FAO: Food and Agricultural Organization of the United Nations
- FEC: friable embryogenic callus
- GA₃: gibberellic acid
- GFP: green fluorescent protein
- GUS: β-glucuronidase
- IBA: indole-3-butyric acid
- IITA: International Institute of Tropical Agriculture, Nigeria
- kb: kilobase, kilobase pairs
- kDa: kilo Dalton
- LIR: large intergenic region
- MES: 2-(N-morpholino)ethanesulfonic acid
- MPB: movement protein
- mRNA: messenger RNA
- NAA: α-naphthalene acetic acid
- NSP: nuclear shuttle protein
- ORF: open reading frame
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDR</td>
<td>pathogen derived resistance</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycole</td>
</tr>
<tr>
<td>PIG</td>
<td>particle inflow gun</td>
</tr>
<tr>
<td>REn</td>
<td>replication enhancer protein</td>
</tr>
<tr>
<td>Rep</td>
<td>replication associated protein</td>
</tr>
<tr>
<td>RIP</td>
<td>ribosome inactivating protein</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light units</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>scaffold attachment region</td>
</tr>
<tr>
<td>sORF</td>
<td>short open reading frame</td>
</tr>
<tr>
<td>ss DNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TGMV</td>
<td>tomato golden mosaic virus</td>
</tr>
<tr>
<td>TMV</td>
<td>tobacco mosaic virus</td>
</tr>
<tr>
<td>TrAP</td>
<td>transactivator protein</td>
</tr>
<tr>
<td>TYLCV</td>
<td>tomato yellow leaf curl virus</td>
</tr>
<tr>
<td>WDV</td>
<td>wheat dwarf virus</td>
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Chapter 1

General Introduction

1.1 Cassava (*Manihot esculenta* Crantz)

Cassava (*Manihot esculenta* Crantz), a member of the family Euphorbiaceae, is a woody perennial shrub. It originated in Latin America and was introduced to Africa in the 16th century by Portuguese traders (Gold, 1994). Today it is cultivated worldwide between 30° south and 30° north of the Equator, providing basic staple food for over 500 million people. Mostly it is grown for its tuberous roots, containing starch up to 85% of their dry weight (Cock, 1982), but also for its protein rich leaves. Cassava has the ability to grow on infertile soils where no other crop could exist without costly external inputs and therefore is the cheapest available food source in many developing countries. It can survive seasonal drought and has the advantage of a highly flexible harvesting time, making it an excellent famine reserve. In Africa it is mostly grown by small-scale farmers as a subsistence crop, providing up to 60% of their daily calorie uptake. An average of 8 t/ha of cassava roots can be produced in a 12-month growing season in Africa (world average is 10 t/ha) and more than 80% of the harvest is used as food (FAO, 1989).

Due to the long culture period of cassava, various insect pests and diseases represent serious challenges to its sustainable cultivation. It has been estimated that around 50% or more of the potential harvest is lost every year (Roca et al., 1992) as a result of viral diseases, bacterial blight disease, mealybugs and green mites or other pests. Furthermore, cassava transport, storage and marketing is complicated by postharvest deterioration. Roots can be kept on growing plants long after the first harvest, but once harvested they must be processed within 2 - 7 days (Wenham, 1995). Another constraint to cassava use is its cyanogenic nature. To prevent cyanide poisoning, cyanogenic glucosides have to be removed and using inadequately processed cassava can have fatal consequences (Akintowa et al., 1993).
Attempts to solve these problems by traditional breeding of cassava plants have had limited success. Traditional breeding in cassava is restricted due to cassava being a highly heterozygous, allopolyploid plant with low natural fertility in many cultivars. In addition the lack of resistance genes in compatible germplasm further complicates breeding improved cassava varieties. Biotechnology, therefore, could provide an alternative approach and could complement efforts in traditional breeding to contribute to a more profitable and sustainable agriculture in Africa by developing improved cassava varieties.

1.2 African cassava mosaic disease

African cassava mosaic disease (ACMD), rated the most serious vector-borne plant disease in Africa (Geddes, 1990), is a severe constraint to cassava cultivation and is an ideal target for an approach combining genetic engineering and traditional breeding. The disease is caused by African cassava mosaic virus (ACMV), which is transmitted by whiteflies (*Bemisia tabaci*) feeding on cassava leaves (Swanson and Harrison, 1994). The viruses are able to persist in their insect vectors for many days or even for the life of the insect, but they do not replicate in their vectors (Shaw, 1996a). ACMV can also be transmitted during propagation via infected tools and planting material, but not via seed (Swanson and Harrison, 1994). Typical symptoms of ACMD are the mosaic bleaching of the leaves, leaf deformation, stunted growth and the loss of storage root formation. ACMD can cause local losses of up to 80%, with losses throughout Africa reaching 36% of total cassava production (Fargette et al., 1988). The only way to protect the plants in the field from being infected is to control the spread of the whiteflies by insecticides but, since whiteflies have overlapping generations and move between cassava and weed hosts, they are not an easy target (Fishpool and Burman, 1994). Frequent spraying, which is environmentally unsound and too expensive for subsistence farmers, would be necessary. Growth reduction of cultivated plants is not directly related to the yield loss due to viral infection; this slows and complicates traditional breeding, as evaluation of new cultivars can only be done at the end of a growing season. To date no completely resistant cultivars have been produced by traditional breeding (Hahn et al., 1980; Thresh and Otim-Nape, 1994), but in Uganda the use of improved cultivars and virus-free planting material have secured and enhanced yields (Bock, 1994; Otim-Nape et al., 1994a; Fargette et al., 1996; Otim-Nape et al., 1997). Disease-free planting material could increase yields two to threefold (Lozano and Booth, 1974); but due to the long growing season of cassava, under high infection pressure, plants
might be reinfected and the benefits of disease-free plants might be short-lived.

### 1.3 African cassava mosaic virus

The African cassava mosaic virus (ACMV, earlier called the cassava latent virus) is a *Begomovirus* of the family *Geminiviridae* (Frischmuth and Stanley, 1991; Lazarowitz, 1992; Bisaro, 1996). Geminiviruses have attracted considerable interest as pathogens attacking many economically important crop plants worldwide. They are transmitted by insect vectors to a variety of monocotyledonous and dicotyledonous plants. The geminivirus family is divided into three subgroups depending on their genome structure and host range (Bisaro, 1996; Shaw, 1996b; Palmer and Rybicki, 1997): The leafhopper-transmitted *Mastreviruses* (formerly subgroup I viruses) like the wheat dwarf virus (WDV), the leaf- and grasshopper-transmitted *Curtoviruses* (formerly subgroup II viruses) like the beet curly top virus (BCTV) and the whitefly-transmitted *Begomoviruses* (formerly subgroup III viruses). *Mastreviruses* and *Curtoviruses* have only a single genomic component of approximately 2.7 kb; *Begomoviruses* may have one or two components of the same size, one of which is dependent on the other for replication.

#### 1.3.1 Genome organization

Only two groups of plant viruses are known to contain DNA genomes: the pararetroviruses and the geminiviruses. Unlike that of the pararetroviruses, the genome of the geminiviruses consists of one or two covalently closed, single-stranded DNA molecules (Stanley and Gay, 1983) denoted DNA A and B. For bipartite viruses, both components were shown to be required for systemic infection (Stanley, 1983).

Comparison of the DNA A and B has revealed a region of approximately 200 nucleotides with almost identical sequences. The 5'-end of this common region has been designated as the zero map unit of DNA A and B (Stanley, 1985). This common region, which is located in the large intergenic region (LIR), is completely different in its sequence among the different geminivirus strains with the exception of a sequence element of about 30 nucleotides. This element can form a hairpin structure consisting of a GC-rich stem and an AT-rich loop (Lazarowitz, 1992). This structure contains the nonamer motif TAATATTAC, which is identical in all geminiviruses (Laufs et al., 1995a).

The seven genes, first identified as open reading frames (ORFs) and named according to their location on the genome and direction of transcription (Stanley and Gay, 1983) and now renamed according to function, have had functions ascribed to
CHAPTER 1. GENERAL INTRODUCTION

Figure 1.1: Genome map of ACMV DNA A and DNA B. The DNA is represented by a thin line with the two arrowheads showing the extent of the large intergenic region (LIR) and the main ORFs are indicated by thick arrows. See text for details on gene names.

them through the construction of mutants and their analysis in infected and in transgenic plants (see Fig. [1.1]). The position of the ORFs relative to the LIR, as well as the polyadenylation signals located on the opposite side of the genome (Buck, 1994), suggest that transcription occurs bidirectionally from the LIR (Townsend et al., 1985). The DNA A contains genes required for encapsidation (CP or AV1, the coat protein gene), for viral replication (Rep or AC1, the replication associated protein gene) and two regulator proteins (TrAP or AC2, a replication transactivator protein and REn or AC3, a replication enhancer protein), required for gene regulation and for efficient replication (Townsend et al., 1985; Etessami et al., 1991; Morris et al., 1991). The DNA B contains two genes required for systemic movement in the host plant (MPB or BC1, responsible for cell-to-cell movement and NSP or BV1, a nuclear shuttle protein) and is probably responsible for symptom production (Morris et al., 1991; von Arnim et al., 1993; Haley et al., 1995; Sanderfoot et al., 1996; Ward et al., 1997). A corresponding protein for AV2 has been reported in tomato leaf curl virus but not for ACMV (Padidam et al., 1996). This ORF is only present in Old World begomoviruses (Hamilton et al., 1984). A major and a minor transcription start site have been mapped upstream of the coat protein and only transcription from the major transcription start site would allow translation of the AV2 protein (Davies et al., 1987). Synthesis of AV2 would require ribosomes to initiate on a very short leader of about 8 nucleotides, which might be expected to result in a rather low level of expression (Kozak, 1983). On the other hand, the coat protein transcript from the major transcription start site has a 160 bp untranslated
leader, containing four AUG triplets, two in phase with the AV2 ORF and two in phase with termination codons upstream of the coat protein (Stanley, 1985). Of these only the coat protein initiation codon shows the preferred A at position -3 (Kozak, 1984), which may be important in its selective recognition (Davies et al., 1987). So far little is known about the regulation of CP expression using the full length promoter.

1.3.2 Viral replication

Geminivirus DNA has been shown to replicate through a circular double-stranded (ds) DNA intermediate (Ikegami et al., 1981) via a rolling-circle mechanism (Lazarowitz, 1992). Apart from a single viral-encoded protein, Rep, which is required for DNA A replication in cis and for DNA B replication in trans, replication relies entirely on the enzymes of the host plant. Known DNA polymerases have no homology with the Rep protein, but Rep may be related to proteins responsible for DNA replication initiation of ss DNA plasmids (Ilyina and Koonin, 1992). The rolling-circle replication is initiated by the Rep protein, which serves as the origin recognition protein and as a site-specific endonuclease (Laufs et al., 1995b; Orozco and Hanley-Bowdoin, 1996). The origin of replication has been mapped to the conserved nonamer motif (TAATATTAC) within the LIR (Heyraud et al., 1993), the stem-loop structure of AVdich is essential for replication as the Rep protein can only nick ss DNA (Fontes et al., 1994). After nicking, the polymerization of viral strand DNA does not only require Rep protein, but also host DNA polymerases, that normally are only active during S-phase of the cell cycle. As geminiviruses infect differentiated cells, they have to re-activate cells to enter cell cycle. Considering that the Rep protein possesses an ATP binding and hydrolyzing activity and that Rep proteins of a wheat ebnaif geminivirus have been shown to form stable complexes with proteins of the retinoblastoma family, it can be speculated that geminiviruses can modulate plant cell cycle (Laufs et al., 1995a; Collin et al., 1996). Once a full-length copy of the viral DNA has been produced, it is cut and ligated to circular ss DNA. This new ss DNA can act as a template for further replication, after complementary-sense DNA synthesis, or can be encapsidated and transported out of the cell.

1.3.3 Gene regulation

During the viral infection cycle, different gene products will be required at different time points and in different quantities. Viral RNAs are polyadenylated and initiate
downstream of TATA box motifs or initiator elements, which indicates that they are transcribed by host RNA polymerase II. The regulation of transcription relies on viral proteins as well as on host factors (Sunter and Bisaro, 1997).

The replication-associated protein is not only responsible for replication, the N-terminus also acts as a primary regulator of its own promoter. It can reduce complementary-sense transcription (Haley et al., 1992; Hong and Stanley, 1995) by binding between the TATA-box and the transcription start site of its own gene (Sunter et al., 1993; Eagle and Hanley-Bowdoin, 1997; Gladfelter et al., 1997). Reduced c-sense transcription from the DNA B was observed for ACMV (Haley et al., 1992), but not for tomato golden mosaic virus (TGMV), even though DNA A and B promoters are similar also in TGMV. A possible explanation for this discrepancy is the presence of a downstream transcription start site in the TGMV DNA B, which allows the MPB gene to escape repression (Sunter et al., 1993).

TrAP is a nuclear protein (Sanderfoot and Lazaro-Avitz, 1995) that transactivates virion-sense gene expression in the Begomovirus group (Sunter and Bisaro, 1991; Haley et al., 1992) at the level of transcription (Sunter and Bisaro, 1992). In transgenic plants containing AV promoter-GUS fusions it could be shown, that TGMV TrAP regulates expression through two different mechanisms: activating the promoter in mesophyll cells and derepressing the promoter in phloem tissues (Sunter and Bisaro, 1997). Also TrAP of ACMV can enhance expression of transgenes under the control of an AV promoter in transgenic plants (Hong et al., 1996). Therefore, TrAP is able to interact with a viral promoter integrated in the plant genome and might also transactivate host genes during the infection cycle. Little is known about the actual mechanism of activation by TrAP, although it was demonstrated that it strongly prefers binding ss DNA over ds DNA (Noris et al., 1996) and that both activities are sequence independent. Further analysis of TrAP revealed a basic N-terminus, an acidic C-terminus and a potential zinc-finger motif, which might mediate DNA binding (Sunter and Bisaro, 1992). The lack of sequence specificity is inconsistent with TrAP's ability to specifically activate v-sense transcription. It has been speculated that specific DNA binding requires post-translational modifications or that TrAP interacts with other proteins of the basal transcription apparatus to activate RNA synthesis (Liu and Green, 1994; Noris et al., 1996; Hanley-Bowdoin et al., 1999). Also REn transactivates virion sense expression at the level of transcription, but the effect is less pronounced (Haley et al., 1992). The mode of action here is not known.
1.4 Strategies for engineering virus resistance

Virus infections in plants can be controlled and suppressed through genes occurring naturally within the plant or the virus. Advances in genetic engineering have led to the development of different strategies to combat virus infections by manipulating these natural processes and engineering a 'pathogen derived resistance' (PDR) (Sanford and Johnson, 1985). The idea originated from the observation of cross-protection: a plant inoculated with one strain may become resistant to a second infection by a related strain of the same virus. This was used as a strategy of biological control, in spite of the risk of yield losses due to the mild infection or of a severe disease produced by the interaction between the mild strain and another virus. As a result of these observations various strategies based on PDR were developed. Genes that confer viral PDR include those for coat proteins (Powell et al., 1986), replicases (Carr and Zaitlin, 1993), movement proteins (Beck et al., 1994; Duan et al., 1997) and for different untranslated nucleic acids (Hemenway et al., 1988; Stanley, 1990; Stanley et al., 1990; Frischmuth and Stanley, 1991; Yepes et al., 1996; Beachy, 1997; Bendahmane and Groenborn, 1997). Usually these strategies are more successful for RNA viruses than for DNA viruses. More recently, new strategies, which are not directly pathogen related, have been developed, using ribozymes or ribosome inactivating proteins (Lodge et al., 1993; Hong et al., 1996; Hong et al., 1997; Yang et al., 1997).

The first PDR was reported in the form of a coat protein-mediated resistance (Powell et al., 1986) that protects the plant from virus infection without the risk of yield reduction. Since then there have been a number of examples of engineered resistance in a variety of crops like tomato, potato, cucumber, rice and against viruses of different families (Beachy et al., 1990; Fitchen and Beachy, 1993). Different mechanisms may lead to coat protein-mediated resistance, but usually the coat protein prevents the disassembly of the viral capsid, an early event of the viral infection (Beachy, 1997). This mechanism is supported by the fact that mostly coat protein-mediated resistance can be overcome by the inoculation of plants with naked RNA (Powell et al., 1986). Most publications report effective resistance to RNA viruses, while coat protein-mediated resistance against DNA viruses is still rare (Kunik et al., 1994; Sinisterra et al., 1999).

Sequences of complete or partial viral replicase proteins can confer a high level of resistance to the virus. Replicase-mediated resistance was first described against tobacco mosaic virus (TMV) in transgenic plants expressing a fragment of the replicase. Although no protein fragment could be detected, plants were resistant. In this case, the active molecule seems to be the produced mRNA (Carr and Zaitlin,
A similar approach provided resistance against a tomato yellow leaf curl virus (TYLCV), which is a DNA geminivirus (Bendahmane and Groenborn, 1997). The mechanism involved in replicase-mediated resistance has not been elucidated, but it is speculated, that the expressed fragment interferes with viral replication. In contrast to coat protein-mediated virus resistance, resistance cannot be overcome by inoculation with naked RNA, since the resistance is at the level of replication and not at the level of uncoating. The replicase-mediated resistance is quite specific.

Movement proteins enable viruses to move from cell to cell or spread systemically in the plant by interacting with the cytoskeleton or plasmodesmata (Koonin et al., 1991; Heinlein et al., 1996; Sanderfoot and Lazarowitz, 1996). Both in the case of DNA and RNA viruses, mutated movement proteins expressed in plants can confer variable levels of resistance against different related viruses (Beck et al., 1994; Duan et al., 1997).

A variety of PDR strategies are based on the expression of nucleic acids which do not encode proteins. One of these approaches is the expression of antisense RNA to reduce the replication of RNA and even DNA viruses. Virus resistance in transgenic model plants ranges from very low to almost total inhibition of viral infection (Hemenway et al., 1988; Yepes et al., 1996; Bendahmane and Groenborn, 1997). Resistance can be due to different mechanisms, either to formation and subsequent degradation of double-stranded RNA, to direct reduction of virus replication or gene expression by high levels of antisense RNA or to gene silencing (Beachy, 1997). Another approach is the use of defective interfering (DI) DNA (Stanley, 1990; Stanley et al., 1990; Frischmuth and Stanley, 1991; Frischmuth et al., 1997a). DI DNAs can be found in plants during natural viral infection. They are partial genomes of the virus which can redirect replication from the viral genomes, thereby decreasing amounts of replicated viral DNA and at the same time reducing spread and symptoms of the virus in the plants.

Other possible strategies for engineering virus resistance in plants include ribozyme-mediated resistance (Yang et al., 1997), where small RNA molecules with a catalytic activity can specifically cleave viral RNA. Another kind of resistance is conferred by an RNA satellite (Harrison et al., 1987). RNA satellites do not have any homology to the virus but they are replicated and encapsidated with the help of the virus. Furthermore, the use of ribosome inactivating proteins (RIPs) has shown to be a successful way of engineering virus resistance in plants (Lodge et al., 1993; Hong et al., 1996; Hong et al., 1997). RIPs are naturally occurring plant proteins, which have antiviral activity against a broad spectrum of plant viruses. They catalyze the depurination of a specific adenine residue of the eucaryotic large
subunit ribosomal RNA, thereby blocking translation (Taylor et al., 1994). The
activation of RIP expression during pathogen attack and release of RIPs into the
cytosol is speculated to lead to local cell death, which prevents replication and spread
of the virus (Bonness et al., 1994).

Engineering ACMV resistance would complement efforts in traditional breeding
and could provide a sustainable solution securing cassava yields. There are indica¬
tions from the model plant Nicotiana benthamiana that genetic engineering of
ACMV resistance is feasible. A number of transgenic model plants with a variable
increase in ACMV resistance have been reported: Constitutive expression of de¬
fective viral DNA (Stanley and Townsend, 1985; Stanley et al., 1990), of antisense
Rep gene (Day et al., 1991), of defective Rep protein (Hong and Stanley, 1996), of
defective movement proteins (von Arnim and Stanley, 1992; Duan et al., 1997), of
coat protein (Kunik et al., 1994) and the virus-induced expression of dianthin in
stably transformed plants (Hong et al., 1996). This suggests that resistant cassava
cultivars using analogous strategies could be obtained in the near future.

1.5 Plant genetic engineering

In order to apply biotechnology in plants, a regeneration system with a compati¬
bile transformation system that allows regeneration of transgenic plants is required.
Plant cells are considered to be totipotent, and thus able to regenerate whole plants
from a single cell. This ability is, however, often limited to certain tissues and devel¬
opmental stages. Further, an efficient transformation system, compatible with the
regeneration system, and a system for identifying and selecting transformed cells are
necessary.

1.5.1 Regeneration

Different regeneration systems have been reported for cassava using a variety of
starting materials (Roca, 1984; Puonti-Kaerlas, 1998). One of the earliest reports is
of a meristem culture (Kartha, 1974) that can be used to obtain planting material
free of viruses and diseases (Kartha and Gamborg, 1975). Now meristems can also
be used to multiply improved cassava varieties by inducing multiple shoot formation
on cytokinin-containing medium (Roca, 1984; Konan et al., 1994). The most reliable
de novo regeneration system for cassava has been through somatic embryogenesis
and cyclic somatic embryogenesis (Stamp and Henshaw, 1982; Stamp and Henshaw,
1987a; Stamp and Henshaw, 1987b) using different auxin-containing media to induce
primary embryogenesis in various cultivars. Maturing somatic embryos can produce
friable embryogenic callus that can be used to establish suspension cultures (Taylor et al., 1996). Alternatively, somatic embryos can be germinated and the developing cotyledons can be used for direct induction of shoot primordia (Li et al., 1998).

1.5.2 Transformation

Gene transfer to plant cells can be obtained by two different systems: direct gene transfer or Agrobacterium-mediated gene transfer. The first plants expressing foreign genes were tobacco plants transformed with Agrobacterium tumefaciens (Barton et al., 1983; Horsch et al., 1985; DeBlock et al., 1987). Agrobacterium has the unique ability of transferring genes to eucaryotes in nature. This has been taken advantage of in genetic engineering by using modified Agrobacterium vectors to transfer genes of interest to plant cells. Direct gene transfer methods, such as microinjection, PEG treatment, calcium-phosphate precipitation or electroporation mostly need specific protocols to regenerate fertile plants from protoplasts (Paszkowski et al., 1984; Paszkowski et al., 1986; Potrykus, 1991). Direct gene transfer through bombarding small DNA coated particles to intact cells can be used to transform organized tissues (Klein et al., 1988). Under optimum conditions particles can penetrate the cell wall and release the DNA for expression and/or integration in the plant genome. J. Finer et al. developed the system further and constructed the Particle Inflow Gun (PIG; (Finer et al., 1992)), which is inexpensive and simple to use. Microtargeting can be particularly used to target a small, precise area of organized tissue (Sautter et al., 1991).

As stable transformation frequencies are low, different marker genes to identify and select stably transformed cells are necessary. Usually an antibiotic resistance gene is introduced to select for transformed cells, allowing the cells containing the transgene to survive and proliferate. In some species (e.g. Dendrobium) selection with antibiotics may not be possible, in this case a visual marker may be used (Chia et al., 1994). β-glucuronidase (GUS) (Jefferson, 1987), luciferase (Ow et al., 1986; Mayerhofer et al., 1995) or green fluorescent protein (GFP) (Sheen et al., 1995) may also be used to monitor transient transformation and to partially optimize transformation conditions. While GUS assays are lethal, both luciferase and GFP expression can be studied in living material.

1.5.3 Cassava transformation and regeneration

Cassava regeneration and transient transformation have been reported using many different methods and target materials, but until recently no compatible systems
had been reported. Now first transgenic cassava plants have been produced using different methods for regeneration and transformation (Li et al., 1996; Schöpke et al., 1996; González et al., 1998). One system makes use of friable embryogenic callus as starting material. The new embryogenic units appear to be of single cell origin, which makes them a good target for transformation (Taylor et al., 1996). After transformation of embryogenic suspensions, transgenic plants of cassava could be regenerated by selection for resistance to paromomycin (González et al., 1998) or using the visual selection system with firefly luciferase (Raemakers et al., 1996). The other system uses cotyledon explants as starting material and regenerates shoots via organogenesis. Since shoots regenerated via organogenesis develop mostly from the cells close to the cut edges of the cotyledons, it makes this system ideal for Agrobacterium-mediated transformation. Transgenic cassava shoots can then be regenerated using hygromycin as a selective agent (Li et al., 1996). The advantage of this system is the short amount of time necessary in in vitro culture, which reduces the risk of somaclonal variation.

1.6 Luciferase reporter genes

Reporter genes are routinely used for transient studies of promoter regulation and gene expression (Ow et al., 1987; Martin et al., 1992; Arias-Garzon and Sayre, 1993; Freeman et al., 1994; Srikantha et al., 1996; Needham et al., 1998). Studies of gene and promoter regulation in ACMV were performed using the GUS reporter gene system (Zhan et al., 1991; Haley et al., 1992), but since then new, improved, reporter systems have been established. One commonly used is the firefly luciferase gene and since recently this can be combined with a Renilla luciferase to measure two different reactions in a single extract.

Firefly luciferase of the common North American firefly Photinus pyralis is the most commonly used of the bioluminescent reporters (Ow et al., 1986; de Wet et al., 1987). The luciferase is a monomeric enzyme of 61 kDa, which catalyzes a two-step oxidation reaction emitting light in the yellow to green region (550-570 nm). ATP, oxygen and luciferin are required as substrates. In order to generate a stable luminescence signal, instead of an initial burst of light, coenzyme A is incorporated in the assay solution to yield maximal luminescence intensity that slowly decays over several minutes. None of these compounds are toxic for plant cells and assays can be performed in vivo. Firefly luciferase assays are very sensitive, allowing the quantification of less than 10⁻²⁰ moles of enzyme (Technical manual, Promega).

*Renilla* luciferase, of the soft coral *Renilla reniformis*, is a monomeric enzyme of
CHAPTER 1. GENERAL INTRODUCTION

31 kDa that catalyzes the oxidation of coelenterazine to yield coelenteramide and blue light of 480 nm (Matthews et al., 1977; Mayerhofer et al., 1995). A limitation of the Renilla luciferase is the presence of a low level of nonenzymatic luminescence, which reduces assay sensitivity by about 10-fold compared with the firefly luciferase assay.

With the Dual-Luciferase™ reporter assay it is possible to measure both luciferases individually within a single system. Since the firefly and the Renilla luciferases are of distinct evolutionary origins and therefore have different enzyme structures and different substrates, it is possible to discriminate between the two reactions. The luminescence of the firefly luciferase can be quenched while at the same time the Renilla luciferase is activated by its substrate. With these two genes, it is now possible to measure the activity of two different promoters in a single rapid assay with high sensitivity.

1.7 Aim of this thesis

African cassava mosaic virus disease is a major threat to African cassava production despite efforts in traditional cassava breeding and genetic engineering of virus resistance in tobacco model plants. Engineering ACMV resistance is of high priority in the genetic improvement of cassava. This will contribute to food security and sustainable agriculture in Africa and at the same time provide important information on both host-pathogen relationships and on virus promoter regulation. The aim of this thesis was to assess and develop new techniques and strategies to produce ACMV resistant cassava.

The properties of regulation of the ACMV promoter make it possible to mimic a hypersensitive reaction by using a finely tuned toxin/anti-toxin system. The two genes chosen for our system are the Bacillus amyloliquefaciens barnase and its specific inhibitor the barstar.

Tobacco was selected as a model plant because of the ease of its transformation (as compared to cassava) and because it has been already used in other ACMV resistance strategies to study ACMV promoter regulation in transiently and stably transformed plants. For promoter regulation studies, the Dual-Luciferase™ reporter assay was used, in order to measure the activity of two different genes (v- and c-sense expression of the ACMV promoter) in a single rapid assay. A transient assay system using transformed cassava protoplasts was developed to test promoter regulation, not just in a model plant, but also in the natural host of the virus. After the promising results of the transient studies, ACMV-promoter-barnase constructs were
transformed into tobacco to assess virus resistance. Transgenic plants were tested using a replication assay to show reduced viral replication in the transgenic plants as compared to wildtype plants.

With the recently developed cassava transformation system, ACMV resistant cassava can be produced. The transferability and adaptability of the current cassava transformation and regeneration were assessed for an African cultivar using the tested ACMV-promoter-barnase constructs. As cassava is grown as many local cultivars it is crucial to adapt transformation to as many cultivars as possible in order to engineer ACMV resistance in local varieties of cassava.
Chapter 2

ACMV Promoter Analysis

2.1 Abstract

African Cassava Mosaic Virus (ACMV) DNA A and DNA B promoters were cloned together with the firefly luciferase and the Renilla luciferase to study the regulation of viral genes. The relative activity of both sides of each promoter was tested in tobacco and cassava protoplasts and for the DNA A promoter also in transgenic tobacco plants. Regulation of the promoters was assessed by cotransforming individual ACMV open reading frames (ORFs) or the complete DNA A with the luciferase constructs. The transactivator protein (TrAP) activates coat protein (CP) and nuclear shuttle protein (NSP) gene expression in both cassava and tobacco protoplasts, but not in transgenic tobacco plants, while DNA A does activate CP expression also in transgenic plants. At the same time DNA A reduces the expression of the replication-associated protein (Rep) but activates the expression of the movement protein (MPB) in tobacco and relatively more so in cassava. These results indicate that by using this promoter, a new virus resistance strategy mimicking a hypersensitive reaction could be designed.

2.2 Introduction

African cassava mosaic virus (ACMV) is a member of the Geminiviridae, a diverse family of plant infectious agents characterized by their circular single-stranded DNA (ssDNA) encapsidated in double icosahedral particles (Hanley-Bowdoin et al., 1999; Lazarowitz, 1992; Stanley, 1985). It belongs to the Begomoviruses, which comprise viruses transmitted by whitefly having a mono- or bipartite genome (DNA A and
CHAPTER 2. ACMV PROMOTER ANALYSIS

Figure 2.1: Genome map of ACMV DNA A and DNA B. The DNA is represented by a thin line with the two arrowheads showing the extent of the large intergenic region (LIR) and the main viral ORFs are indicated by thick arrows. See text for details on gene names.

DNA B). ACMV replicates in the nuclei of host cells through double stranded DNA intermediates via a rolling circle mechanism and it recruits most proteins of the replication machinery from its hosts. The coat protein and all viral functions required for replication are encoded on the DNA A: CP, the coat protein (Townsend et al., 1985); Rep, the replication-associated protein (Etessami et al., 1991), TrAP (transactivator protein) and REn (replication enhancer protein), regulator proteins (Haley et al., 1992; Etessami et al., 1991). DNA A, therefore, is capable of autonomous replication and encapsidation but is unable to infect plants systemically (Townsend et al., 1986). NSP (nuclear shuttle protein) and MPB (moving protein), the two genes positioned on DNA B, provide functions for virus movement (von Arnim et al., 1993; Haley et al., 1995). Both genomic components, DNA A and B, are required for infectivity (Stanley, 1983).

The arrangement of the open reading frames (ORFs) of the DNA A and B (Fig. 2.1) shows that they are expressed in a bidirectional manner (Townsend et al., 1985). The ORFs on both DNA A and B are arranged similarly with divergent transcription units separated by a large intergenic region that is highly conserved between the two DNAs (Stanley and Gay, 1983). This segment also contains a ~30 nt sequence with the potential to form a stem-loop structure that is conserved among bipartite geminiviruses and has been associated with the start of rolling circle replication (Lazarowitz, 1992). Fragments of DNA A (nucleotides 2759 to 282) and DNA B (nucleotides 2705 to 581) have been shown to act as inducible promoters with relatively low basal activity (further on referred to as AC and AV promoter for...
the DNA A in c- and v-sense and BC and BV promoter for the DNA B promoters in c- and v-sense) (Zhan et al., 1991; Haley et al., 1992). Studies of promoter regulation have shown that the AV, the BV and the BC promoters can be activated by TrAP while the AC promoter is repressed by its own gene product (Haley et al., 1992; Hong and Stanley, 1995; Sunter and Bisaro, 1997). Both activation and repression occur at the level of transcription (Sunter and Bisaro, 1992; Sunter et al., 1993). Rep is known to bind between the TATA-box and the transcription start site, thereby probably hindering its own transcription by interfering with the RNA polymerase (Haley et al., 1992; Sunter et al., 1993; Hong and Stanley, 1995). Up to date there is little information available on the molecular mechanisms responsible for promoter activation by TrAP (Haley et al., 1992; Sunter and Bisaro, 1997).

All published data on promoter regulation has been obtained from transient expression experiments using only the uidA (GUS) reporter gene (Jefferson et al., 1987). This has the disadvantage that only one promoter could be studied in each experiment. The use of the firefly and the Renilla luciferase now permit the discrimination between the two reactions in a single extract and thereby make it possible to measure two different promoter activities in one experiment (Hannah et al., 1998). This is due to the fact that the firefly and the Renilla luciferases are of distinct evolutionary origins and therefore have different enzyme structures and different substrates. Moreover, both luciferases can be detected at low levels in a fast and simple assay.

Here we report the analysis of the activities of the ACMV promoters by quantifying transient expression of the firefly and Renilla luciferase reporter genes using the Dual-Luciferase™ reporter assay for the first time in plant cells. We examined the expression from both sides of the ACMV DNA A and DNA B promoters and the regulation of these promoters by Rep and TrAP and the complete DNA A, simulating a viral infection. To study viral promoter activity in planta, transgenic plants containing the luciferase constructs were infected with a geminivirus and analyzed. The characteristics of the regulation of the bidirectional ACMV promoter indicate that it might be a useful tool for the creation of a virus resistance strategy mimicking hypersensitivity.
CHAPTER 2. ACMV PROMOTER ANALYSIS

2.3 Materials and methods

2.3.1 Plant material

Cassava (*Manihot esculenta* Crantz) was maintained as embryogenic suspension cultures. These were established from friable embryogenic calli of cultivar TMS 60444. The suspensions were grown axenically in 25 ml of modified liquid SH medium (Schenk and Hildebrandt, 1972; Taylor et al., 1996), at 30°C under continuous low light in a growth chamber (Infors Incubator HT04) on a rotary shaker (90 rpm). All suspensions were subcultured once a week.

Tobacco, *Nicotiana tabacum* L. cv. Petit Havanna Str-r1 (SR1; (Maliga et al., 1973)), was maintained as axenic shoot cultures. These were grown in vitro at 26°C with a 16/8 h photoperiod on half-strength MS medium without growth regulators (Murashige and Skoog, 1962). SR1 tobacco seedlings used for *Agrobacterium*-mediated transformation were grown axenically on filter paper wetted with sterile water.

2.3.2 Bacterial strains

*E. coli* strain XL1-Blue was purchased from Stratagene (La Jolla, CA, USA). *A. tumefaciens* strain GV3101, containing helper plasmid pGV2260 (Deblaere et al., 1985) was kindly provided by B. Tinland, Zürich.

2.3.3 Construction of plasmids

The plasmid pACMVA is a partial repeat (1.3 mer) of DNA A (West Kenyan isolate 844 (Stanley and Gay, 1983)) in pBS KS+ (Stratagene). The plasmid pACMVB is a tandem repeat of DNA B (West Kenyan isolate 844 (Stanley and Gay, 1983)) in pAT153. The cloning of pACMVA and pACMVB has been described (Klinkenberg et al., 1989). Both plasmids were kindly provided by T. Frischmuth, Stuttgart. The ACMV ORFs Rep and TrAP constructs (Halev et al., 1992) were kindly provided by B. Morris, New Zealand.

All DNA manipulations were performed according to standard procedures (Maniatis et al., 1982). Nucleotide numbering of the ACMV genome refers to pJS092 and pJS094 (Stanley and Davies, 1985). DNA A, DNA B and names of the open reading frames are according to suggested nomenclature (Davies and Stanley, 1989). In case the gene function is known, the name of the gene product is indicated. The bidirectional ACMV promoters were produced by PCR amplification of ACMV DNA using primers ACMVc (GGAA -2755- GCTTTTTGACCAAGTCAATTGG -2776)
and ACMVs (300-GTGGTACCCACTATTGCGCACTAGC -267) for the short version of the DNA A promoter, primers ACMVe and ACMVI (GGGGTACC -440- AGCCCTGATAACTGAG -425) for the longer DNA A promoter. The DNA B promoter (Zhan et al., 1991) was amplified with primers ACMVBL (ATCCCGT -581- CAATGTATATACTTCC -564) and ACMVBR (GTTAGGCCATGG -2265- TTCAACACTTTTGAGTATAAGC -2286). All versions of pACMV were obtained by introducing the ACMV promoters as KpnI/MunI fragments in between the two tobacco scaffold attachment regions (SAR; (Breyne et al., 1992)) in the backbone of pGluChi (Bliffeld et al., 1999). The firefly luciferase ((Ow et al., 1986); kindly provided by G. Neunhaus-Urb, Basel) together with a nos terminator was introduced as an NcoI/XbaI fragment under the control of the AC or the BC promoter, while the Renilla luciferase from pPCV702 (Mayerhofer et al., 1995) was introduced together with a nos terminator as a blunt-ended HindIII fragment under the control of the AV or BV promoter.

A short and a longer version of the DNA A promoter were cloned. The short version of the promoter ends at the major transcription start site of the coat protein while the longer version also includes the minor transcription start site of the coat protein. Transcription from the major transcription start site of the longer version gives rise to a 160 nt-long leader fragment upstream of the Renilla luciferase gene. The plasmids were called pACMVs, pACMVleader and pACMVB depending on the inserted promoter (see also Fig. [2.2] and Fig. [2.3]). Each of the ACMV promoter constructs created by PCR amplification was sequenced to ensure that no mutations had been introduced. As positive controls both the Renilla and firefly luciferase were separately cloned under the control of a truncated 35S promoter and a 35S terminator ((Odell et al., 1985); kindly provided by S. Brunner, Zürich) rendering plasmids pLucpos and pRenpos. To reduce the luciferase expression, thereby making measurement more convenient, the truncated 35S promoter consisted of nucleotides 1 to 93 only.

In order to transform plants with Agrobacterium, the luciferase construct containing the short DNA A promoter was introduced into pNC1 as a complete I-SceI-fragment. pNC1 is a pCambia1300 backbone (Cambia, Australia) with a pMCS5 (MoBiTec, Germany) polylinker containing an I-SceI site. This plasmid, pNCsrl, was then electroporated (Mattanovich et al., 1989) into GV3101 (pGV2260) resulting in strain AgNCsrl.
Figure 2.2: **Schematic representation of luciferase expression cassette.** Luciferase expression cassette showing the position of the cloned ACMV promoter (the short or the longer version of the DNA A promoter or the DNA B promoter) between the *Renilla* and the firefly luciferase genes. Both luciferase genes carry a *nos* terminator. The whole expression cassette is flanked by scaffold attachment regions (SAR). The bar underneath the map marks the position of the ffluc probe used for Southern hybridization analysis.

### 2.3.4 Protoplast isolation and transformation

**Tobacco mesophyll protoplasts**

Mesophyll protoplasts were isolated from *in vitro*-grown tobacco plants. Leaves were cut into 1 cm$^2$ pieces and digested overnight in PCN1 (Golds et al., 1993) containing 1% Cellulase Onozuka R10 (Yakult Pharmaceuticals Ind. Co., Ltd., Japan), 0.5% Macerozyme R10 (Yakult Pharmaceuticals Ind. Co., Ltd., Japan) and 1 drop Tween80 (Nagy and Maliga, 1976). The protoplast suspension was filtered through a 100 μm steel mesh sieve. After collecting the protoplasts by floating on PCN1 they were washed twice with W5 (Menczel et al., 1981) and then incubated in the dark at 4°C for 1-2 hours. Samples of 250,000 to 500,000 protoplasts were resuspended in transformation buffer (15 mM MgCl$_2$, 0.1% MES 0.5 M mannitol, pH 5.8) and transformed with 20 μg of DNA using PEG (Negrutiu et al., 1987). Following transformation, protoplasts were incubated in PCN2 (Golds et al., 1993) at RT in the dark.

After 24 hours protoplasts were harvested by centrifugation and resuspended in 100 μl passive lysis buffer for the Dual-Luciferase™ Reporter assay (Promega, Catalys AG, Switzerland). Extracts were stored at -80°C for up to one month.

**Cassava protoplasts from embryogenic suspension cultures**

Approximately 1 g of TMS 60444 embryogenic suspension culture was digested in the dark during 20 h at 28°C on a shaker (30 rpm). The enzyme solution and the washing solution have been described (Sofiari et al., 1998). The digest was then filtered through a 50 μm mesh sieve and 10 ml washing solution were added through the filter. The filtrate was distributed in sterile tissue culture tubes and
centrifuged at 70 g (30%) for 7 min in a Hettich table centrifuge (Universal II). The supernatant was removed and the pelleted protoplasts were washed twice and resuspended in 10 ml washing solution. The protoplasts were counted, stored and transformed as described for tobacco protoplasts. The protoplasts were incubated at RT in the dark in TM2G (Shahin, 1985) for 24 hours prior to harvesting and resuspending in passive lysis buffer.

2.3.5 Agrobacterium-mediated seedling transformation of tobacco

Tobacco seedlings grown for 8 days were transformed by vacuum infiltration as described (Rossi et al., 1993) using an overnight culture of AgNCsIr grown in 2 ml YEB (Vervliet et al., 1975) containing the appropriate antibiotics. The seedlings were cocultivated for 3 days on solid MS medium and then washed in 10 mM MgSO₄. They were then placed on MS medium containing 0.1 μg/ml α-naphtalene acetic acid, 1μg/ml benzylamino purine, 25 μg/ml hygromycin, 500 μg/ml cefotaxime and 500 μg/ml vancomycin. Seedlings were transferred to fresh plates every week. After 7-8 weeks, shoots could be collected from single calli and transferred to MS medium without selection. After multiplying each plant line, rooted plantlets could be transferred to soil or be kept as in vitro cultures.

2.3.6 Dual-Luciferase™ reporter assay

Protoplast or leaf disc extracts were thawed and cell debris was collected by centrifugation. Aliquots of 50 μl of Luciferase Assay ReagentII (Promega, Catalys AG, Switzerland) were pre-dispensed into luminometer tubes before adding 10 μl of extract and mixing well with a pipette. Measurements of 5 seconds each were performed with a luminometer (Lumat LB 9507, EG & G Berthold, Switzerland) with dual injectors. After measuring the luciferase activity, in 'relative light units' (RLU), Stop and Glo™ Reagent was delivered into the tube by automatic injection. Measurement of the Renilla luminescence was started after a two second delay. For each experiment, background luciferase activity from protoplasts transformed with a vector without luciferase genes was subtracted throughout. Protein concentration, estimated using a Bio-Rad protein assay (Bio-Rad Laboratories, USA) as described (Bradford, 1976), was used to normalize each measurement. All resulting values were standardized to the positive control consisting of protoplasts transformed with pLucpos and pRenpos.
2.3.7 Southern blot analysis

Total DNA from transgenic and untransformed control tobacco was extracted using the NucleonPhytopure kit (Amersham Life Science, England) as described by the manufacturer. Aliquots of 10 μg DNA were digested with NcoI, resulting in a single cut in the T-DNA. After electrophoresis, DNA was transferred to a positively charged nylon membrane (Amersham Pharmacia Biotech, UK) by blotting overnight as described (Sambrook et al., 1989). After a short washing of the membrane, the DNA was covalently bound to the membrane by exposure to UV light (UV Stratalinker 1800, Stratagene). The membrane was prehybridized with 10 ml DIG Easy Hyb™ (Roche Molecular Biochemicals, Switzerland). After one hour the prehybridization solution was replaced with fresh hybridization solution including 20 ng DIG-labeled probe and hybridization took place at 42°C overnight. DIG-labeling of a 330 bp fragment of the luciferase gene was performed according to the PCR DIG Probe Synthesis Kit™ standard protocol (Roche Molecular Biochemicals, Switzerland). After removal of unspecific hybridization and incubation in blocking buffer, the anti-DIG antibodies were added. Following washing and equilibration, the DNA side of the membrane was overlaid with substrate solution (CDPstar™, Roche Molecular Biochemicals, Switzerland). The damp membrane was sealed in a plastic bag, fixed in a film cassette and exposed to X-ray film (BIOMAX MR™, Kodak, Rochester NY, USA) for 1 hour at 37°C. The film was developed with a developing machine (AGFA, Curix 60).

2.3.8 Virus infection of transgenic plants and assay of viral DNA replication

Transgenic plants were grown in soil in a controlled growth chamber and inoculated with A. tumefaciens containing the viral DNA of ACMV or of Sida Golden Mosaic Virus (SiGMV, Colombian isolate) as described (Stanley et al., 1990). All A. tumefaciens strains were kindly provided by T. Frischmuth, Stuttgart, and have been described (Stanley et al., 1990; Frischmuth et al., 1997b). Samples of leaves of three different ages (leaf 1, leaf 3 or 4 and leaf 5 or 6; numbered from bottom to top) were collected 24 days after infection. Luciferase extracts of infected and non infected leaves were measured.
CHAPTER 2. ACMV PROMOTER ANALYSIS

2.4 Results

2.4.1 Expression of firefly and Renilla luciferase genes under the control of ACMV promoters

The ACMV DNA A and DNA B promoters were cloned between two different luciferase genes. This allowed the study of the activation/down-regulation of both sides of the bidirectional promoters in a single experiment. The firefly luciferase gene was cloned under the control of the AC promoter (c-sense expression), which in the DNA A is the promoter of the replication associated protein and in the DNA B the promoter of the protein responsible for long distance movement. The Renilla luciferase was cloned under the control of the V1 promoter (v-sense expression), which in DNA A is the promoter of the coat protein and in the DNA B the promoter of the nuclear shuttle protein. For the DNA A promoter the Renilla luciferase gene was cloned under the control of two different versions of the V1 promoter: a short version, which includes only the major transcription start site at genome position 278 (construct pACMVs), and a longer version (construct pACMVleader; see Fig.[2.3]), which enables transcription from the major and the minor transcription start site (genome position 378; (Davies et al., 1987)). Transcription from the major transcription start site of pACMVleader results in an RNA containing a 160 nt leader sequence upstream of the Renilla luciferase ORF, which in this construct replaces the ACMV coat protein ORF. In this leader two AUGs (at 286 and 305) are upstream of the initiation codon of the coat protein gene at nucleotide 446. These additional AUGs are in frame with a small ORF V2, beginning 8 nucleotides downstream of the major transcription start site and overlapping the ORF of the coat protein. The existence of the ORF V2 encoded protein for ACMV has not been reported to date, but a corresponding protein for AV2 has been reported in tomato leaf curl virus (Padidam et al., 1996).

In different experiments variable background levels for firefly luciferase (100 - 1000 RLUs) and Renilla luciferase (1500 - 30000 RLUs) were observed. Within one experiment, these background levels were quite constant and expression levels were regarded as significant only when they were at least 3 times above this background. Background levels were substracted from all values before standardization against total protein content. The basal levels of the firefly luciferase from pACMVs and pACMVleader were around 8000 RLUs. The Renilla luciferase from pACMVs gave rise to values of about 50000 RLUs, while the activity from pACMVleader was higher, about 145000 RLUs. The firefly luciferase activity of pACMVB was comparable to that from pACMVs, but the Renilla luciferase activity was on average
Figure 2.3: Constructs used for ACMV promoter studies in comparison with original ACMV DNA. Luciferase expression cassettes compared with ACMV DNA, showing the position of the cloned ACMV promoter (the short or the longer version of the DNA A promoter or the DNA B promoter) between the Renilla and the firefly luciferase genes. Both luciferase genes carry a nos terminator. The whole expression cassette is flanked by scaffold attachment regions (SARs).

25 times higher (table 2.1). In our experimental system the Renilla and firefly luciferase genes under the control of the same promoter produced RLU’s that varied by a factor of maximum 2.5 (data not shown) and similar results have been reported for mammalian cells (Promega User Manual); therefore, similar RLU’s for Renilla and firefly luciferase reflect similar promoter strengths. All luciferase activities measured for any of the ACMV promoters were at least 100-fold and up to 2000-fold lower than activities obtained for the same luciferases fused to a truncated (-1 to -93) 35S promoter.
Table 2.1:
Basal luciferase activities of pACMVs, pACMVleader and pACMVB

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<td>100%</td>
<td>100%</td>
<td>195%</td>
</tr>
<tr>
<td>Relative b</td>
<td>100%</td>
<td>730%</td>
<td>100%</td>
</tr>
</tbody>
</table>

aLuciferase activities of pACMVleader and pACMVB were standardized to luciferase activities of pACMVs.
bV-sense Renilla luciferase activities were standardized to the respective c-sense firefly luciferase activities.

2.4.2 Effect of the expression of viral ORFs on the activity of the luciferase genes under the control of ACMV promoters

The constructs pACMVs or pACMVB were cotransformed into tobacco protoplasts, either with equimolar amounts of the Rep construct (ACMV Rep ORF under the control of a 35S promoter), TrAP construct (ACMV TrAP ORF under the control of a 35S promoter), ACMV DNA A or with carrier DNA. All experiments were repeated 4 to 6 times and the results are summarized in Fig. [2.4].

Cotransformation with the TrAP construct increased v-sense expression (Renilla luciferase) of pACMVs 16-fold and c-sense expression (firefly luciferase) 10-fold; cotransformation with the Rep construct reduced c-sense expression about 35-fold but did not affect v-sense expression. Cotransformation with the complete DNA A resulted in an almost 50-fold increase of v-sense expression and an approximately 2-fold reduction of c-sense expression. Even in the cases of lowest c-sense expression, firefly luciferase activity remained significantly above background levels, indicating that also under these conditions, the promoter maintains a basal activity. The regulation of the B promoter by the TrAP construct was similar; the Rep construct, however, showed a stronger negative effect on v-sense expression (Renilla luciferase) and a slightly weaker negative effect on c-sense expression. The most striking difference was observed for cotransformation with the DNA A, which led to a strong activation of c-sense (20-fold) and v-sense (16-fold) expression from the DNA B promoter.
Figure 2.4: **Effect of the expression of ACMV ORFs on the activity of firefly and Renilla luciferase under the control of ACMV promoters in tobacco protoplasts.** Luciferase constructs pACMVs and pACMV B were assayed in transient for luciferase activity in tobacco SR1 protoplasts following cotransformation with the Rep construct, the TrAP construct or the complete DNA A. A value of 100% was assigned to the activity of each promoter construct alone. Columns represent the resulting luciferase activity as a percentage of the basal activity and are the means of four to six independent experiments; error bars represent standard deviation.

Analogous experiments were also performed with cassava protoplasts (see Fig. [2.5]). The results were similar in as far as TrAP functioned as an activator of both c- and v-sense expression with DNA A and B promoters; the highest increase in activity, however, was observed with the DNA BC promoter in presence of TrAP (57-fold). Like in tobacco protoplasts, Rep reduced c-sense expression in general and most strongly again in the case of the AC promoter (35-fold). The cotransformation with DNA A resulted in a 20-fold increase of v-sense expression (Renilla luciferase) but had no significant effect on the c-sense expression (firefly luciferase). The results using pACMVB in cassava were again different from those in tobacco. In cassava the increase of c-sense expression was much stronger (35-fold) as compared with the 6-fold increase of v-sense expression, while in tobacco both luciferase activities increased to a similar extent.
Figure 2.5: Effect of the expression of ACMV ORFs on the activity of firefly and Renilla luciferase under the control of ACMV promoters in cassava protoplasts. Luciferase constructs pACMVs and pACMVB were assayed in transient for luciferase activity in cassava SR1 protoplasts following cotransformation with the Rep construct, the TrAP construct or the complete DNA A. A value of 100% was assigned to the activity of each promoter construct alone. Columns represent the resulting luciferase activity as a percentage of the basal activity and are the means of four to six independent experiments; error bars represent standard deviation.

Furthermore pACMVs was cotransformed with the TrAP and Rep constructs together using different amounts of DNA for each construct (1 or 10 μg each, see Fig. 2.6]). As these experiments were performed separately, results were not combined with experiments where Rep and TrAP were cotransformed individually. Transformation with increasing amounts of the Rep construct reduced c-sense expression, while v-sense expression was still enhanced even if TrAP was cotransformed at very low amounts (1 μg TrAP vs. 10 μg Rep construct). In the case where equal amounts of both constructs are transformed, the increase of v-sense expression together with the decrease of c-sense expression resembles a cotransformation experiment using DNA A.
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2.4.3 Analysis of a short and a longer form of the DNA A promoter

The constructs with two different segments of the ACMV DNA A promoter pACMVs and pACMVleader were cotransformed into tobacco protoplasts together with the complete DNA A. A summary of the experiments is shown in Fig. [2.7]. When cotransformed with DNA A, the increase in v-sense expression (Renilla luciferase) from the short version was much higher (58-fold) than the increase from the longer version of the promoter (6-fold). The reduction of c-sense expression (firefly luciferase) was 4- to 10-fold for both the short and longer version of the DNA A promoter.

Figure 2.6: Effect of the combined expression of ACMV ORFs on the activity of firefly and Renilla luciferase under the control of ACMV promoters in tobacco protoplasts. Luciferase construct pACMVs was assayed in transient for luciferase activity in tobacco SR1 protoplasts following cotransformation with different combinations of the Rep and TrAP constructs. A value of 100% was assigned to the activity of pACMVs alone. Columns represent the resulting luciferase activity as a percentage of the basal activity and are the means of four independent experiments; error bars represent standard deviation.
Figure 2.7: ACMV ORFs regulate luciferase expression from the short and longer version of the ACMV DNA A promoter in tobacco protoplasts. pACMVs and pACMVleader were cotransformed with the complete DNA A into tobacco protoplasts. Luciferase activities were determined and are expressed as a percentage of the activity of each promoter construct alone. Columns represent the mean values of four experiments; error bars represent the standard deviation.

2.4.4 Production of transgenic tobacco plants containing pACMVs and pACMVleader

To test whether the results from the transient expression experiments in protoplasts could be confirmed for stably integrated genes, transgenic tobacco plants containing pACMVs or pACMVleader were produced by Agrobacterium-mediated transformation. Resistance to hygromycin was used to select transgenic plants. The transgenic nature of 10 independent lines was confirmed by Southern blot analysis (see Fig.[2.8]) and luciferase assays of leaves. Of these 10 transgenic lines, 6 contained the short version of the DNA A promoter, while 4 contained the longer version of the promoter. All lines contained 2 - 10 copies of the T-DNA. Two of these lines showed a 3 : 1 segregation pattern (shr 6 and shr 11), indicating a single locus integration. All plants were grown as in vitro cultures as well as in the greenhouse, where they
Figure 2.8: Southern analysis of ACMV luciferase transgenes in tobacco. Southern blot of NcoI restriction fragments hybridized to the fluc probe.

produced seeds normally.

**Regulation of the luciferase genes under the control of the ACMV promoter in transgenic tobacco**

Mesophyll protoplasts were isolated from transgenic plants and transformed with the Rep or TrAP construct or with the complete DNA A. Protoplast extracts were analyzed with the Dual-Luciferase™ assay and standardized with Bradford assays, but the variation of the relative values between plants and experiments was high. A representative experiment with some of the plant lines and a wildtype SR1 tobacco (cotransformed with pACMV s or with pACMV s linearized outside of the expression units) is shown in table 2.2. Surprisingly, c-sense expression (firefly luciferase), in the transgenic plants, was much stronger than v-sense expression (Renilla luciferase) or at least similar (slr 9). This is in contrast with transient experiments, where v-sense expression was always stronger. Also in contrast to the earlier cotransformation experiments in protoplasts, TrAP did not function as an activator in protoplasts from transgenic plants. Only when the complete DNA A was used for transformation, an increase of v-sense expression could be found, while the c-sense expression...
**CHAPTER 2. ACMV PROMOTER ANALYSIS**

### Table 2.2:
Luciferase expression (in RLUs) of protoplasts from transgenic tobacco plants after transient transformation with ACMV ORFs

<table>
<thead>
<tr>
<th>plant line</th>
<th>reporter gene</th>
<th>constructs used for transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rep</td>
</tr>
<tr>
<td>slr 1</td>
<td>firefly luciferase</td>
<td>200000</td>
</tr>
<tr>
<td>slr 1</td>
<td><em>Renilla</em> luciferase</td>
<td>6000</td>
</tr>
<tr>
<td>slr 9</td>
<td>firefly luciferase</td>
<td>1870</td>
</tr>
<tr>
<td>slr 9</td>
<td><em>Renilla</em> luciferase</td>
<td>3150</td>
</tr>
<tr>
<td>slr 11</td>
<td>firefly luciferase</td>
<td>13000</td>
</tr>
<tr>
<td>slr 11</td>
<td><em>Renilla</em> luciferase</td>
<td>3500</td>
</tr>
<tr>
<td>llr 7</td>
<td>firefly luciferase</td>
<td>16300</td>
</tr>
<tr>
<td>llr 7</td>
<td><em>Renilla</em> luciferase</td>
<td>3100</td>
</tr>
<tr>
<td>llr 5b</td>
<td>firefly luciferase</td>
<td>2100</td>
</tr>
<tr>
<td>llr 5b</td>
<td><em>Renilla</em> luciferase</td>
<td>1600</td>
</tr>
<tr>
<td>wt</td>
<td>firefly luciferase a</td>
<td>660</td>
</tr>
<tr>
<td>wt</td>
<td><em>Renilla</em> luciferase</td>
<td>7800</td>
</tr>
<tr>
<td>wt, linear</td>
<td>firefly luciferase b</td>
<td>520</td>
</tr>
<tr>
<td>wt, linear</td>
<td><em>Renilla</em> luciferase</td>
<td>16000</td>
</tr>
</tbody>
</table>

*Wildtype protoplasts were cotransformed with pACMVs and the corresponding ACMV ORF.*
*Wildtype protoplasts, cotransformed with linearized pACMVs and the corresponding ACMV ORF.*

remained unaffected. This increase varied from 3- to 50-fold between different experiments. This same effect could also be observed when using linearized pACMVs for cotransformation studies, although here TrAP slightly activated *Renilla* luciferase expression in some experiments (data not shown). As variation between experiments was much higher than between different lines, the degree of activation could not be correlated to the gene copy number. Also, in contrast to cotransformation experiments described above, the expression in both v- and c-sense from the longer version of the DNA A promoter was not notably altered by transformation with the DNA A or the TrAP construct. When transformed with the TrAP construct even a slight reduction of both luciferases could be detected in protoplasts of transgenic plants containing the longer version of the DNA A promoter.
**Infection of transgenic tobacco plants**

Seeds from selfed transgenic plants were collected, germinated and infected with ACMV. Infected leaves of three different stages (leaf 1, leaf 3 or 4 and leaf 5 or 6) were analyzed for luciferase activity 24 days after infection, even though plants showed no ACMD symptoms. Ten infected plants of five different lines were tested using uninfected plants of the same line as controls. Of these 50 plants only three plants showed an activation of the *Renilla* luciferase expression after viral infection (srl6-4, srl9-13 and srl11-8). In order to assess the amount of viral DNA present, DNA of infected plants was isolated and analyzed in a Southern blot. No viral DNA could be detected in any of the plants tested. Therefore, new plants were germinated and infected with SiGMV (Colombian isolate). All plants showed typical mosaic bleaching of leaves, indicating an infection rate of 100%. Three infected - and three non-infected plants of 5 different lines were tested. All of these plants showed very high luciferase activities but *Renilla* activities were hardly above background even if the plant was infected (data not shown). A relative activation of *Renilla* luciferase activity and down-regulation of firefly luciferase activity (2- to 5-fold) could be observed in all tested lines.
2.5 Discussion

The large intergenic region of the geminivirus genome contains cis-acting elements that are important for the regulation of viral gene expression and viral replication. A regulated, bidirectional promoter is situated within this intergenic region (Stanley and Gay, 1983; Townsend et al., 1985; Haley et al., 1992; Eagle and Hanley-Bowdoin, 1997; Orozco et al., 1998). Previous studies on geminivirus promoter regulation investigated mainly promoter-GUS fusions, testing the transcriptional activity for each direction of the promoter separately (Zhan et al., 1991; Morris et al., 1991; Brough et al., 1992; Haley et al., 1992; Sunter et al., 1993; Zhan et al., 1993; Gröning et al., 1994; Hong and Stanley, 1995; Sunter and Bisaro, 1997; Gooding et al., 1999; Ruiz-Medrano et al., 1999). Overall, the results obtained with Begomovirus promoters revealed a similar regulation pattern: the TrAP protein was found to induce v-sense transcription while Rep down-regulates c-sense transcription (Haley et al., 1992). However, details of basal promoter strength and the degree of activation or repression by viral proteins varied considerably. It remained unclear whether these variations reflect differences of the respective assay systems (e.g. plant material, incubation time of transformed protoplasts, exact promoter sequences, etc.) or, alternatively, differences in the replication cycle of the respective viruses. Tomato golden mosaic virus (TGMV), for example, starts replicating after 18 - 24 hours in protoplasts (Brough et al., 1992), while in ACMV or beet curly top virus (BCTV) infected cells, replicating DNA can only be detected 2 - 3 days after infection (Townsend et al., 1986; Briddon et al., 1989).

We intended to use the regulation mode of the ACMV promoter for a novel strategy for engineered virus-inducible virus resistance, which required a more detailed knowledge of the relative strengths of the two promoter directions in absence and presence of viral gene products. In order to study simultaneously transcription from both sides of the promoter (further on referred to as AC and AV promoter for the DNA A and BC and BV promoter for the DNA B promoters), the promoter fragments were fused to the firefly luciferase gene in c-sense orientation (AC and BC promoters) and to the Renilla luciferase gene in v-sense orientation (AV and BV promoters). Since several start sites have been reported (Davies et al., 1987) for the DNA A v-sense transcription, we also tested both a short promoter, covering only the start site upstream of the AV2 gene, and a longer version of the promoter, covering all the start sites upstream of the coat protein gene (see Fig.[2.3]). The two different luciferases can be tested in the same reaction mixture and should, therefore, allow a precise comparison of promoter activities. Using reference constructs containing either one luciferase under the control of a truncated (1-93) 35S
CHAPTER 2. ACMV PROMOTER ANALYSIS

promoter, we could establish that similar amounts of light units are produced by both luciferases from these constructs, indicating that light units can be directly related to promoter activity.

An additional advantage of the luciferase reporter genes is the shorter half-life (for Renilla luciferase only reported in mammalian cells) of both proteins (and possibly mRNAs) compared to the previously used GUS reporter proteins (Thompson et al., 1991; Martin et al., 1992). This allows a better evaluation of gene induction or repression.

In tobacco or cassava protoplasts transformed with plasmid DNA, the strongest expression from any of the introduced genes under the control of an ACMV promoter was still considerably lower than the expression obtained with a truncated 35S promoter, which again was about 3-fold weaker than from the full-length promoter (data not shown). Thus, in our assay system, the ACMV promoters are about 300- to 6000-fold weaker than the 35S CaMV promoter, in contrast to previous reports, which stated a 10- to 40-fold lower activity of the ACMV promoter (Zhan et al., 1991; Hong and Stanley, 1995). The activity of a TGMV AV promoter on a replicon was reported to be even 60- to 90-fold stronger than that of a 35S promoter and without replication, the activity of the TGMV promoter was still comparable to that of the 35S promoter (Brough et al., 1992).

In contrast to earlier reports, where the basal expression from the AC promoter was considerably stronger than from the other three promoters, we find that in transient assays c-sense expression is always lower than v-sense expression (see table 2.1). In transgenic plants, on the other hand, the AC promoter leads to higher expression rates than the AV promoter, although the differences of luciferase activity between separate plant lines and experiments varied strongly. This could be due to the different availability of host factors in the whole plant or due to the higher stability of the firefly luciferase compared to the Renilla luciferase. To date nothing is known about the half-life of Renilla luciferase in plant cells.

For the repressing effect of the Rep and the activating effect of TrAP we found quantitative differences to published data. Both, the 10- to 20-fold repression of c-sense expression by Rep and the 10- to 15-fold stimulation of v-sense expression by TrAP are more similar to values previously found for TGMV (Gröning et al., 1994; Sunter et al., 1993) than to the much smaller values reported for ACMV (Haley et al., 1992; Hong and Stanley, 1995). These results are in agreement with a model of early/late gene regulation: since Rep is needed for replication, it will be expressed early in the infection cycle but repressed later on. On the other hand, expression of the coat protein (CP protein), which will be required in high amounts later on in
the infection cycle once the replicated DNA is ready for encapsidation, is activated by TrAP (Stanley, 1985).

Rep also repressed BC expression 2- to 5-fold, but, as expected, had no or only small effects on v-sense expression. Surprisingly, TrAP also activated DNA A c-sense expression by about 10-fold, a property not previously recognized. Activation by TrAP was also strong for the DNA B promoter in both orientations in tobacco protoplasts, but in cassava protoplasts a high disparity of activation between the two DNA B promoters was observed: the activation of the BC promoter was much higher than that of the BV promoter. Otherwise, the two protoplast systems reacted similarly. The regulation of the DNA B promoter in cassava is in accordance with the role of the two DNA B proteins: NSP has been reported to bind newly replicated ssDNA and to move the DNA out of the nucleus (Sanderfoot et al., 1996) while the MPB forms endoplasmatic reticulum-derived tubules which extend through the cell wall to the next cell (Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996; Ward et al., 1997). The nuclear shuttle protein expressed individually, is localized in the nucleus, however when MPB is coexpressed, it is relocalized to the cell periphery (Sanderfoot and Lazarowitz, 1996; Lazarowitz and Beachy, 1999). The results obtained for cassava suggest a regulation of targeting NSP: early in the infection cycle more NSP will be produced which will favor localization in the nucleus. Later, after activation of c-sense expression, the excess of MPB will redirect NSP, together with the bound viral DNA to the periphery of the cell, and facilitate its movement across the cell wall. This model does not incorporate the encapsidation step which, however, is not necessary for systemic movement of ACMV in the plant (Sanderfoot and Lazarowitz, 1996); it is only necessary for the transmission via the whitefly vector (Lazarowitz, 1992).

Since the regulation of the DNA B promoter is different in cassava and tobacco it follows, that not only the viral proteins but also cellular factors play a role in the regulation of the ACMV promoters. Species-specific variation in the availability of such factors could account for the lower activation of the BV promoter in cassava protoplasts as compared to tobacco protoplasts. This further shows the importance of testing the obtained results in the natural host of the virus.

To mimic a viral infection, reporter gene constructs were cotransformed with the complete ACMV DNA A, which should express regulatory proteins under viral expression signals and viral control. DNA A proved to be a more efficient inducer of v-sense expression than TrAP and a less efficient repressor of c-sense expression than Rep for the DNA A promoters. It appears that the repressive effect of the DNA A is dominant for the AC promoter, while the inducive effect is dominant for the AV
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This dominance was also apparent in cotransformation experiments with different ratios of Rep and TrAP, where a small amount of TrAP expressing plasmid was sufficient to suppress the repressing effect of Rep on v-sense expression (see Fig. 2.6). The strength of the activation of v-sense expression is surprising since in the cotransformation of single genes, these genes were under the control of the 35S promoter, which should produce much higher levels of protein. Possibly the complete DNA A contains additional activating sequences, which either allow more efficient TrAP production or which produce additional proteins or protein variants with activation potential. Another possibility is that the replication associated protein induces cell cycle factors that could increase the expression of the reporter gene (Ruiz-Medrano et al., 1999). In contrast to the differential effect on the DNA A promoter, both DNA B promoters were strongly stimulated by DNA A, suggesting that the stimulatory effect of TrAP (and/or other proteins) is dominant for both the BC and BV promoters.

The longer version of the DNA A promoter was activated much less efficiently and even considering the 3-fold higher basal activity, the final expression levels of Renilla luciferase (v-sense expression) remained lower than those from the short promoter. According to published transcript analyses, the major transcription start site is located upstream of the AUG codon of the coat protein ORF, implying that Renilla luciferase fusions to the downstream coat protein ORF, like in the construct pACMValleader, would be less efficiently translated due to the presence of upstream, out-of-frame start codons. It would, therefore, be expected that expression from the short promoter is more efficient than from the longer version of the promoter. The observed lower activation of Renilla luciferase expression could be explained by assuming that only transcription at the major start site is activated by DNA A. According to this model, using pACMValleader more of the longer mRNA (containing the 160 bp leader sequence) is produced while the level of the short mRNA, transcribed from the minor transcription site, should not be affected. Since translation of the longer mRNA is probably not very efficient due to out-of-frame start codons in the leader sequence upstream of the CP ORF (Fütterer and Hohn, 1992), it will not give rise to a high level of luciferase activity. On the other hand, with pACMVs, transcription of the leaderless mRNA will be enhanced and translation can take place efficiently, resulting in a much higher level of luciferase activity. We have not been able to detect the corresponding mRNAs by RNase protection assay (results not shown). This is probably due to the instability of the luciferase mRNAs. It therefore remains unclear which transcripts are produced with the different expression constructs in our assay system.
For the envisaged virus resistance strategies the use of a promoter integrated in the plant genome is necessary. The studies performed with the free plasmid in protoplasts might be close to the situation of a virus infecting a cell, but the regulation of a promoter integrated in the chromatin could be very different. In parallel experiments we compared the effects of Rep, TrAP or DNA A on reporter expression from cotransformed or integrated copies of pACMV or pACMVleader. Interestingly, the absolute values of these transformation experiments are very different in individual plant lines. For example, line slr 1 shows a much higher basal c-sense expression (firefly luciferase) than v-sense expression (Renilla luciferase) in mesophyll protoplasts, while line slr 9 exhibits a slightly higher basal v-sense than c-sense expression. It seems that expression is not only regulated by the ACMV promoter but that also the integration site plays a role in the regulation of gene expression, despite the presence of SARs.

After cotransformation with DNA A the relative increase of expression of the wildtype is higher than that of all the other upregulated transgenic plants. The stronger increase of v-sense expression in the cotransformed wildtype plants could be due to a different regulation of the integrated virus promoter versus a virus promoter localized in a plasmid. At the same time this could provide an explanation for the fact that unlike in wildtype protoplasts, no increase of expression can be detected after transformation with the TrAP construct. Using the longer version of the promoter, activation of v-sense expression could be observed neither when using TrAP nor DNA A for cotransformation. Already in the transient assay the increase of v-sense expression from the longer promoter upon transformation with DNA A is lower than that from the short version. As this increase of v-sense expression from pACMV is 4-fold lower in the transgenic plants as compared to the transient assay, the increase from pACMVleader in transgenic plants is hardly above the basal activity.

During the many repetitions performed in the course of these experiments, the results involving transactivation of reporter activity particularly showed a relatively large variability. Insufficient control of DNA structure during the experiments could be one reason for this variability and possibly for differences between our data and those reported previously. Importance of DNA structure was first suggested by results obtained with protoplasts from transgenic tobacco plants harboring the same reporter genes as used for the transient assays. DNA curvature has been implicated in control of replication and possibly transcription for the wheat dwarf geminivirus (Gutierrez et al., 1995; Gooding et al., 1999), where the expression of a reporter gene from introduced plasmid DNA was different from that from in planta-generated
replicons. The authors speculate, that the effect could be due to the activation states of the chromatin or the different methylation states. Such features would almost certainly be different on a supercoiled plasmid and on integrated DNA, as the latter will probably be associated with nucleosomes differently. To verify this, we transformed protoplasts with reporter DNA that was linearized outside of the expression units. In these experiments, expression from linear DNA was hardly activated by TrAP protein, whereas this process was efficient with circular DNA (see table 2.2).

Also the activation of Renilla luciferase expression with DNA A was less efficient using linear DNA than circular DNA. It can also be speculated, that additionally a viral protein, e.g. Rep, interacting with host transcription factors (Hanley-Bowdoin et al., 1999; Liu et al., 1999; Ruiz-Medrano et al., 1999), is required for maximum virion sense expression, even though the protein itself is not an activator. In this case transformation with TrAP might not be sufficient for activation when the promoter is integrated in the genome.

As a logical step after transient assays transgenic plants were infected with ACMV. Unexpectedly, ACMV was not able to infect tobacco SR1 plants, therefore, another geminivirus, the Sida Golden Mosaic Virus (SiGMV) was used for infection studies. Typical symptoms could be detected on infected plants after 2 to 3 weeks, indicating a systemic infection. However, quantification of luciferase activities in different leaves of infected plants revealed that Renilla expression was only slightly above background, even in infected plants. Possibly, SiGMV is not related closely enough to ACMV to activate its promoter properly. The viability of pseudorecombinants produced by reassortment of genome components of ACMV, Squash Leaf Curl Virus or Tomato Golden Mosaic Virus implies that trans-acting functions are interchangeable (Stanley et al., 1985; Lazarowitz, 1991). Nevertheless, it has been shown that viruses of the New World have a more limited ability to recognize and functionally interact with DNA of other geminiviruses than viruses from the Old World (Frischmuth et al., 1993b), which is in agreement with our results.

An approach in which the unique properties of this promoter were exploited has already been published (Hong et al., 1996). There expression of a ribosome inactivating protein was induced upon virus infection. Since the AV promoter is slightly leaky, as shown by using the GUS reporter system (Hong et al., 1996), this might lead to abnormal or reduced growth, when using a toxic gene under the control of the AV promoter. Therefore, in addition to using the upregulation of the AV promoter, we propose to also use the downregulation of the AC promoter: the AC promoter could regulate the expression of the inhibitor of a toxin which is under the control of the AV promoter. As long as no infection occurs, more
inhibitor than toxin will be produced. Only if the cell is infected, the balance will be shifted towards more toxin production. Consequently a hypersensitive reaction could be mimicked thereby protecting the plants from viral infection. The results of our studies on ACMV promoter regulation are of practical significance for designing new virus resistance strategies in the future.
Chapter 3

Developing a new ACMV resistance strategy by mimicking a hypersensitive reaction using the barnase and barstar ORFs

3.1 Abstract

Barnase is an unspecific ribonuclease of *Bacillus amyloliquefaciens* which has a specific inhibitor, the barstar. Here the RNase activity of the barnase was exploited to engineer resistance to a DNA plant virus, the African cassava mosaic virus in transgenic *Nicotiana tabacum* SR1. For this purpose the barnase ORF was cloned under the ACMV virion-sense promoter, which is *trans*-activated by the TrAP, a viral protein. Additionally, the barstar was introduced under the control of the ACMV complementary-sense promoter to counteract basal expression of barnase. Upon viral infection the ratio of barnase/barstar would be expected to shift in favor of the barnase due to the viral protein TrAP, resulting in local cell death before the virus can spread to adjacent cells. A replication assay using leaves of transgenic plants compared with wildtype plants could show a 3- to 100-fold reduction of viral replication in transgenic leaves.
3.2 Introduction

African cassava mosaic disease (ACMD) has been rated one of the most important and devastating diseases of cassava in Africa (Geddes, 1990). It was first reported in 1884 (Warburg, 1894) and several serious outbreaks have been reported since then (Thresh et al., 1994). At present, a particularly severe epidemic of the disease is spreading in central and eastern Africa (Gibson et al., 1996) causing local losses of up to 80%, while losses throughout Africa reach 36% of total cassava production (Fargette et al., 1988). Cassava (Manihot esculenta Crantz) is a major food crop in Africa, grown for its tuberous roots containing up to 85% starch (dry weight) and for its protein-rich leaves. It is the basic staple food for over 200 million people in Sub-Saharan Africa, where the majority of cassava is produced by small-scale farmers on a subsistence basis. Conventional breeding to control African cassava mosaic disease by inter- and intraspecific crosses with plants having a natural resistance but a limited agricultural value, is still difficult due to the low fertility of many local varieties (Hahn et al., 1980; Thresh and Otim-Nape, 1994). Genetic engineering is a powerful tool to complement these efforts by extending the gene pool for useful gene sources over species barriers and by engineering single, desirable traits precisely.

ACMD is caused by a whitefly-transmitted virus of the family Geminiviridae: the African cassava mosaic virus or ACMV (Briddon et al., 1998). The ACMV genome is composed of two circular single-stranded DNA molecules, DNA A and B (see Fig. [3.1]). The larger of the two components, DNA A (2779 bp), encodes the coat protein (CP or AV1) and all viral functions required for viral replication (Rep
or AC1, replication associated protein; TrAP or AC2 and REn or AC3, regulator proteins). DNA A is therefore capable of autonomous replication and encapsidation (Townsend et al., 1986), but incapable of moving from cell to cell in the host plant. The second component, DNA B (2724 bp), encoding two proteins (MPB or BC1 and NSP or BV1) that are involved in cell-to-cell movement in the plant (von Arnim et al., 1993; Haley et al., 1995), is required for a full systemic infection. Transcription occurs in a bidirectional manner from the large intergenic region (LIR), which is highly conserved between DNA A and DNA B.

Increasing knowledge of the functions of ACMV genes and their regulation has led to the development of virus resistant transgenic model plants. Transgenic constitutive expression of defective viral DNA (Stanley and Townsend, 1985; Stanley et al., 1990), of antisense Rep gene (Day et al., 1991), of defective Rep protein (Hong and Stanley, 1996), of defective movement proteins (von Arnim and Stanley, 1992; Duan et al., 1997), of coat protein (Kunik et al., 1994) or the virus-induced expression of dianthin in stably transformed plants (Hong et al., 1996) has shown to confer geminivirus resistance. As shown with the ribosome-inactivating protein dianthin, the regulatable ACMV promoter can be used for the controlled expression of highly toxic genes that otherwise would be lethal or lead to abnormal development of the transgenic plants, thus limiting their effectiveness as antiviral agents (Hong et al., 1996).

The unique properties of the regulation of the ACMV promoter should make it possible to mimic a hypersensitive reaction by using a finely tuned toxin/anti-toxin system. It is known that a basal low level of transcription takes place from both sides of the ACMV promoter (Hong et al., 1996). Therefore, basal expression of the toxin has to be counteracted with basal expression of the anti-toxin. Upon viral infection of transgenic plants, expression of the toxin under the control of the AV promoter would be activated specifically in virus infected cells, thus overriding the effect of the anti-toxin under the control of the AC promoter.

Barnase, the ribonuclease produced by *Bacillus amyloliquefaciens* (Mariani et al., 1990; Mariani et al., 1992), and its specific inhibitor, barstar, could be applied in the aforementioned virus resistance strategy. Due to the high toxicity of the barnase, further down-regulation of its production might prove necessary. This could be achieved by introducing additional short open reading frames (sORF), upstream of the barnase gene, to down-regulate its translation (Fütterer and Hohn, 1992; Ruan et al., 1996; Mize et al., 1998).

We report here the cloning and transformation of such constructs into *N. tabacum* SR1 in order to analyze the influence of the regulated barnase expression on plant
development, to assess the reduction of viral replication in transgenic plants transiently and to study the effect of the transactivation of the barnase gene upon viral infection.
3.3 Materials and methods

3.3.1 Plant material

Tobacco, *Nicotiana tabacum* L. cv. Petit Havanna Str-r1 (SR1) (Maliga et al., 1973), was maintained as axenic shoot cultures. These were grown *in vitro* at 26°C with 16 h light per day on half-strength MS medium without growth regulators (Murashige and Skoog, 1962). Tobacco seedlings used for *Agrobacterium*-mediated transformation were grown on filter paper wetted with sterile tap water for 8 days prior to use.

3.3.2 Bacterial strains

*E. coli* strain XL1-Blue was purchased from Stratagene (La Jolla, CA, USA). *A. tumefaciens* strain GV3101, containing helper plasmid pGV2260 (Deblaere et al., 1985) was kindly provided by B. Tinland, Zürich.

3.3.3 Construction of plasmids

Plasmid pACMV A is a partial repeat (1.3mer) of DNA A (West Kenyan isolate 844 (Stanley and Gay, 1983)) in pBS II KS+. The cloning of the 1.3mer of DNA A has been described (Klinkenberg et al., 1989).

All DNA manipulations were performed according to standard procedures (Maniatis et al., 1982). Nucleotide numbering of the ACMV genome refers to pJS092 and pJS094 (Stanley and Davies, 1985). DNA A and names of the open reading frames are according to suggested nomenclature (Davies and Stanley, 1989), unless the function of a gene is known. In this case the name of the gene product is indicated. The bidirectional ACMV promoter was produced by PCR amplification of ACMV DNA A using primers ACMVc (GGAA -2755- GCTTTTTGACCAAGTCAATT-GG -2776) and ACMVs (300-GTGGTACCCACTATTGCGCACTAGC -267). The ACMV promoter was introduced as a *Kpn*I/*Mun*I fragment between the two tobacco scaffold attachment regions (SAR) (Breyne et al., 1992) in the backbone of pGluChi (Bliffeld et al., 1999). The barstar ORF (Hartley, 1988) was cloned under the control of the AC promoter as an *Nco*I/*Pst*I fragment, while the barnase ORF (Hartley, 1988) (both kindly provided by J. Fütterer, Zürich) was introduced as a blunt-end fragment into the blunt *Spe*I site under the control of the AVI promoter. Both ORFs were cloned together with a 35 S terminator. The hygromycin resistance cassette was cloned as a blunt end fragment downstream of the barstar gene,
resulting in plasmid pACMVbb (see also Fig. [3.2]). Correct cloning of inserts into the vector was confirmed by restriction digests and sequencing.

In order to regulate barnase expression also at the translational level, various short ORFs were cloned between the ACMV promoter and the barnase gene (Ruan et al., 1996). For the introduction of these sORFs it was necessary to subclone the HindIII fragment of pACMVbb into pGN35Smuc+ (kindly provided by G. Neuhaus, Basel). Primers sORF1 (CGAACGGATCAACCAT(G/A)GCCGGCGAT(A/G)TCAGCTAGCT) and sORF2 (AGCTGA(C/T)ATCGCCGGC(T/C)AT- GGTTGATCCGTTCCGGTAC) were annealed and ligated to the KpnI and SacI sites of the HindIII fragment. This resulted in four different sORF constructs depending on which of the degenerated oligonucleotides was introduced. These sORFs could be discriminated by an NcoI site (present in the primer with G in the first degenerate position) and an EcoRV site (present in the primer with A in the second degenerate position; see also table 3.1).

As a positive control the firefly luciferase and the Renilla luciferase were cloned under the control of a 35S promoter and a 35S terminator (kindly provided by S. Brunner, Zürich) rendering plasmid pLucpos and pRenpos respectively (see Chapter 2). To reduce the luciferase activity and thereby make measurement more convenient, the 35S promoter only contained nucleotides 1 to 93. As a non-replicating negative control, pACMV ΔAC1, a part of a 1.3mer of the DNA A, without the fragment containing the DNA A from bp 2732 over 2779/1 until bp 1868, in which most of the Rep ORF (bp 1680 to 2756) is excised, was used. This plasmid was kindly provided by H. Wohlwend, Zürich.

In order to transform plants with Agrobacterium, the barnase construct was introduced into pNC1 as a complete I-SceI-fragment. pNC1 is a pCambia1300 backbone with a pMCS5 polylinker (MoBiTec, Germany) containing an I-SceI site. The resulting plasmid, pNCbb, was then electroporated (Mattanovich et al., 1989) into

<table>
<thead>
<tr>
<th>NcoI site</th>
<th>EcoRV site</th>
<th>no. of sORFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>one (6 aa)</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>two, overlapping</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>none</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>one (7 aa)</td>
</tr>
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Figure 3.2: Schematic representation of barnase expression cassette. Barnase expression cassette showing the position of the cloned ACMV DNA A promoter between the barnase and the barstar ORFs. The barnase ORF contains the intron from rice tungro bacilliform virus; both ORFs carry a 35S terminator (35S). An hpt ORF under the control of a 35S promoter (35SP), with a nos terminator (nos!) is included as a selectable marker. The whole expression cassette is flanked by scaffold attachment regions (SAR). The bar over the map represents the position of the barnase probe used for Southern hybridization analysis.

GV3101 (pGV2260) resulting in strain GV*bainase for agroinoculation of seedlings.

3.3.4 Tobacco mesophyll protoplast isolation, transformation and regeneration

Mesophyll protoplasts were isolated from in vitro-grown tobacco plants. Leaves were cut into 1 cm² pieces and digested overnight in PCN1 (Golds et al., 1993) containing 1% Cellulase Onozuka R10 (Yakult Pharmaceuticals Ind. Co., Ltd., Japan), 0.5% Macerozyme R10 (Yakult Pharmaceuticals Ind. Co., Ltd., Japan) and 1 drop Tween80 (Nagy and Maliga, 1976). The protoplast suspension was filtered through a 100-μm steel mesh sieve. After collecting the protoplasts by floating on PCN1 they were washed twice with W5 (Menczel et al., 1981) and then incubated in the dark at 4°C for 1 - 2 hours. Samples of 250,000 to 500,000 protoplasts were resuspended in transformation buffer (15 mM MgCl₂, 0.1% MES 0.5 M mannitol, pH 5.8) and transformed with 20 μg of DNA using PEG (Negrutiu et al., 1987). PCN2 (Golds et al., 1993) was added to the transformed protoplasts before they were incubated at RT in the dark.

After 24 hours protoplasts were harvested by centrifugation and resuspended in 100 μl passive lysis buffer for the Dual-Luciferase™ reporter assay (Promega, Catalys AG, Switzerland). Extracts were stored at -80°C for up to one month.

For stable transformation, protoplasts were washed after the transformation and resuspended in 0.5 ml of PCN2. Aliquots of 4.5 ml warm PCN2 containing 0.6% seaplaque agarose were carefully mixed with the transformed protoplasts in a 6 cm-Falcon petri dish. After solidification of the agarose, the protoplasts were cultured...
in the dark for 24 hours and in semi-darkness for the following week. The agarose disc was then cut in half and each half immersed into 30 ml of medium A (Caboche, 1980) containing the appropriate selective agent. Bead cultures were incubated on a rotary shaker with 80 rpm until resistant colonies could be seen after 3-4 weeks. One week later, these were placed on solid MS morpho medium (Spangenberg et al., 1990) where shoot development took place after 2-6 weeks. Fully developed shoots were transferred to MS medium without hormones. After establishment of a root system, plantlets could be kept as sterile cultures or transplanted to soil in the greenhouse.

3.3.5 *Agrobacterium*-mediated seedling transformation of tobacco

Eight-day-old tobacco seedlings were transformed by vacuum infiltration (Rossi et al., 1993) using an overnight culture of GVbar nase grown in 2 ml YEB (Vervliet et al., 1975) containing the appropriate antibiotics. The seedlings were cocultivated for 3 days on solid MS medium (Murashige and Skoog, 1962) and then washed in 10 mM MgSO₄. They were then placed on MS medium containing 0.1 μg/ml NAA, 1μg/ml BA, 25 μg/ml hygromycin, 500 μg/ml cefotaxime and 500 μg/ml vancomycin. Seedlings were transferred to fresh plates every week. After 7-8 weeks, shoots could be collected from single calli and transferred to MS medium without selection. After multiplying each plant line, rooted plantlets could be transferred to soil or kept as *in vitro* cultures.

3.3.6 Dual-Luciferase™ reporter assay

Protoplast extracts were thawed and cell debris was collected by centrifugation. 50 μl of Luciferase Assay ReagentII (Promega, Catalys, Switzerland) were pre-dispensed into luminometer tubes before adding 10 μl of extract and mixing well with a pipette. Measurements were performed with a luminometer (Lumat LB 9507, EG & G Berchthold, Switzerland) with dual injectors during 5 seconds each. After measuring the luciferase activity, in 'relative light units' (RLU), Stop and Glo™ Reagent was delivered into the tube by automatic injection. Measurement of the *Renilla* luminescence was started after a two second delay. For each experiment, background luciferase activity from protoplasts transformed with a vector without luciferase genes was subtracted throughout. Protein concentration, estimated using a Bio-Rad protein assay as described (Bradford, 1976), was used to normalize each measurement. All resulting values were standardized to the positive control consisting of
protoplasts transformed with pLucpos and pRenpos.

3.3.7 Molecular analysis

PCR

Total DNA from transgenic tobaccos and an untransformed control was extracted using the NucleonPhytopure kit (Amersham Life Science, England) as described by the manufacturer. PCR reactions (Mullis and Faloona, 1987) were performed in a final volume of 50 µl using 1 µg of plant DNA and 5 units of Taq polymerase (QIAGEN, Hilden, Germany). Primers were added to a final concentration of 1 µM each and dNTPs (Boehringer Mannheim, Germany) were used at a concentration of 250 µM. PCR cycles consisted of a 1 min denaturation at 95 °C a 1 min annealing at around 60 °C (depending on GC content and length of primer) and an elongation period of 1 min at 72 °C. After 25 cycles, amplified products were separated by agarose gel electrophoresis. In order to detect the barnase gene, a 600 bp fragment of the barnase and of part of the ACMV promoter was amplified using primers ACMVF (5'-CCACTATATACCTACAGGC-3') and barnaseF (5'-CATGTTCGTCCGCTTGCC-3'). To verify the presence of the hygromycin resistance cassette, a 550 bp fragment was amplified with primers 35Shpt5 (5'-GAAAAGGAAGGTGGCTCC-3') and hpt3 (5'-AATAGGTCAGGCTCTCGC-3').

Southern blot

Total DNA from transgenic tobaccos and an untransformed control was extracted using the NucleonPhytopure kit (Amersham Life Science, England) as described by the manufacturer. Aliquots of 10 µg of this DNA were digested either with PstI, expected to give rise to a fragment containing the complete barnase-ACMV promoter-barstar (around 1.5 kb) or HindIII, which should result in a fragment of around 700 bp and a single cut in the T-DNA leading to the plant DNA. After agarose gel electrophoresis, DNA was transferred to a positively charged nylon membrane (HybondN, Amersham) by blotting overnight as described (Sambrook et al., 1989). After a short washing of the membrane, the DNA was covalently bound by exposure to UV (UV Stratalink 1800, Stratagene). The membrane was prehybridized at 42°C with 10 ml DIG Easy HybTM (Roche Molecular Biochemicals, Switzerland). After one hour the prehybridization solution was replaced with fresh hybridization solution including 20 ng DIG-labeled probe and hybridization took place at 42°C overnight. DIG-labeling of the DNA was performed according to the PCR DIG Probe Synthesis Kit™ standard protocol (Roche Molecular Biochemicals,
Switzerland). A 600 bp fragment of the barnase and part of the ACMV promoter was amplified. After removal of unspecific hybridization and an incubation in blocking buffer, the anti-DIG antibodies were added. Following equilibration, the DNA side of the membrane was overlayed with substrate solution (CDPstarTM, Roche Molecular Biochemicals, Switzerland). The damp membrane was sealed in a hybridization bag, fixed in a film cassette and exposed to X-ray film (BIOMAX MR™, Kodak, Rochester NY, USA) for 1 hour at 37°C. The film was developed with a developing machine (AGFA, Curix 60).

3.3.8 Replication assays

Transformation of tobacco leaf discs by particle bombardment

Two hours before bombardiment leaf discs of 2 cm in diameter were placed on MS medium, pH 5.8, supplemented with 10% sucrose and solidified with 0.8% agar. Two μl DNA (0.1 μg/μl) were added into a tube containing 25 μl of gold-particle suspension (50 μg/μl) and DNA was precipitated on particles using CaCl2, spermidine and ethanol (Klein et al., 1988). Aliquots of 5 μl were used for bombardment. A nylon baffle grid (500 μm mesh) was placed between the filter and the target at 10 cm from the filter, in order to reduce gas impact on the tissue. The target was placed at 12.5 cm from the filter. The chamber was evacuated to 100 mbar and the particles were accelerated by a helium jet generated by 6-7 bar pressure for 50 milliseconds. Two hours after bombardment leaf discs were transferred to MS medium without growth regulators containing 2% sucrose and incubated in the light at 24°C for six days.

Analysis of ACMV DNA replication

Total DNA from bombarded leaf discs was isolated as described (Soni and Murray, 1994). Aliquots of 5 μg of total DNA from each sample were digested with MluI/DpnI and with MluI/DpnI/MboI. The restricted samples were electrophoresed through 1% agarose gels and transferred to nylon membranes (Hybond N, Amersham). ACMV replicated DNA was detected by hybridization to an AV1 probe labeled with digoxigenin-11-dUTP. Labeling, hybridization and chemiluminescent detection were performed according to the instructions of the manufacturer (Roche Molecular Diagnostics). pACMVA, restricted with MluI, resulting in a hybridizing fragment of 2.7 kb, was used as a positive control.
3.4 Results

3.4.1 Expression of active barnase can be enhanced transiently in tobacco protoplasts

In order to test the activity of the barnase and to assess the ACMV promoter regulation, tobacco protoplasts were cotransformed with pLucpos, pACMVbb and with or without complete DNA A, thereby mimicking a viral infection. As expected, the luciferase activity in protoplasts in which the barnase expression was activated (cotransformed with DNA A), was much lower (30% of the luciferase activity in protoplast in which barnase was not activated) than in protoplasts cotransformed only with the barnase and the luciferase constructs. Due to the high toxicity of the barnase, it was expected, that additional downregulation of barnase expression might be necessary. Different sORFs (see table 3.1) were introduced upstream of the barnase to reduce translation. Four different constructs containing sORFs were tested. Also with these constructs a reduction of luciferase activity could be observed upon cotransformation with the complete DNA A, although this reduction was slightly less marked (45 - 50% of the luciferase activity in protoplasts in which barnase was not activated).

3.4.2 Analysis of transgenic tobacco plants containing a regulatable barnase ORF

Tobacco plants were transformed with pACMVbb via Agrobacterium-mediated seedling transformation. Resistant plants were analyzed by PCR, amplifying a 600 bp fragment of the barnase and part of the ACMV promoter or a 550 bp fragment of the hygromycin resistance cassette. PCR results revealed that even though all plants contained the hygromycin gene, no plants contained the barnase gene. In order to examine this partial integration of pACMVbb, plants were analyzed additionally by Southern blot. Of the analyzed plants only one plant contained the complete fragment liberated by PstI (sbb17), but at least 4 plants contained rearranged inserts with only part of the barnase gene present. Since transformation efficiency seemed low and only few plants could be regenerated, a new approach was tested, transforming plants with pACMVbb containing different sORFs. These sORFs have been shown to down-regulate gene expression in mammalian, yeast and plant cells 8- and 20-fold (Füitterer and Hohn, 1992; Ruan et al., 1996; Mize et al., 1998). Transgenic tobacco plants were produced via PEG-mediated protoplast transformation. Upon analysis with PCR, transformed plants regenerated from protoplasts
Figure 3.3: **Southern analysis of barnase transgenes in tobacco.** Southern blot of HindIII (H) and PstI (P) restriction fragments hybridized to the barnase probe. Total DNA of transgenic plants digested with PstI gives rise to a fragment containing the complete barnase-ACMV promoter-barstar fragment (around 1.5 kb), while digests with HindIII result in a fragment of around 700 bp and a single cut in the T-DNA leading to the plant DNA. Names of plant lines are according to the respective sORF (see table 3.1).

were negative for amplification of the barnase gene but positive for amplification of the hygromycin resistance gene. When the same plants were analyzed by Southern blots, most showed rearrangements when hybridized with the barnase probe. Only 6 plants that contained the complete PstI fragment were found (see Fig. [3.3]) in addition to sbb17, which had been transformed earlier via Agrobacterium. The 7 plant lines were grown and multiplied as in vitro shoot cultures and developed normally except for plants of line sbb17. On older leaves, these plants developed necrotic lesions that gradually spread over the complete leaf and also to younger leaves. Plant growth was reduced after the first lesions were visible, 3 - 6 weeks after subculturing.
Figure 3.4: Leaf replication assay. Total DNA of shot leaves of plants sbbl7, +5 and wildtype tobacco was isolated and equal amounts were digested either with MluI/DpnI or with MluI/DpnI/MboI. Viral replication occurred only when leaves were shot with DNA A and was reduced in transgenic plants.

3.4.3 Viral replication in transient assays is reduced in transgenic plants

Plants containing the complete barnase and barstar genes and untransformed control plants were used for replication assays to test the inhibition of viral replication in these plants. Leaf discs were transformed by particle bombardment with pACMV A, pACMVΔAC1, a replication defective DNA A, or with a control vector without viral DNA A. Standard amounts of total DNA, isolated after 6 days, were digested with MluI/DpnI or with MluI/DpnI and MboI. Taking advantage of the methylation sensitivity of DpnI (active only if DNA is methylated) and MboI (active only if DNA is not methylated) it is possible to distinguish between input DNA (methylated) and de-novo synthesized viral DNA (not methylated). In the restriction digest without MboI a viral band was detected only if viral replication occurred in the leaf. This band disappeared upon additional digestion with MboI, confirming the viral nature of this DNA. Input DNA was completely digested in both cases. Compared to replication in wildtype plants (100%), viral replication in leaves of plants sbbl7, sORF++5 and sORF++11 transformed with pACMV A was much lower at 13%, 30% and less than 1% respectively (see representative example in Fig.[3.4]). No replication occurred in leaves transformed with pACMV ΔAC1 or with an empty vector. A repetition of this experiment could confirm the reduced viral replication in transgenic plants.
3.5 Discussion

Barnase, an RNase of *Bacillus amyloliquefaciens*, is able to efficiently trigger cell death and can be specifically inhibited by the barstar in plants (Mariani et al., 1990; Mariani et al., 1992). As expression of a dianthin gene under the control of the regulatable ACMV AV promoter has been shown to confer ACMV resistance (Hong et al., 1996), our strategy, using the ACMV promoter together with the barnase and barstar to confer virus resistance, should be feasible. The system tested here is more advanced, since beside the barnase, under the control of the ACMV AV promoter, also the barstar, under the control of the ACMV AC promoter, plays an integral role, inhibiting the low level of produced barnase in non-infected plants.

The fact that the barnase is active and that its expression can be activated was demonstrated with transient experiments in protoplasts using the luciferase as a reporter gene. The luciferase activity in protoplasts cotransformed with pACMVshyg and pLucpos was higher than that of protoplasts cotransformed with pACMVshyg, pLucpos and ACMV DNA A, indicating that barnase expression was activated by the DNA A and that the increase of barnase production did actually reduce RNA. This could be seen for barnase constructs both with and without sORFs.

Stable transformation of tobacco plants with these constructs proved to be difficult even though, initially, the regeneration frequency of hygromycin resistant plants seemed normal. However, when analyzed by PCR, no fragment could be amplified from any of the regenerated plants. Only when analyzed by Southern blot could the presence of the ACMV promoter and the barstar ORF be confirmed. Secondary structures of the ACMV promoter sequence and the barstar ORF with high melting temperatures of 61°C and 58°C respectively, might be the reason for the false negative results of the PCR. Since these secondary structures did not melt at the annealing temperatures required by the primers chosen for PCR analysis, primer annealing and subsequent polymerization were very inefficient and no PCR product could be detected.

Only one plant containing the complete barnase-ACMV promoter-barstar fragment could be identified from the first 8 transformation experiments. It has been shown for tomato golden mosaic virus that repression of v-sense transcription is not maintained in unorganized tissue (Sunter and Bisaro, 1997). This could explain the low yield of plants containing the complete insert: most plants containing a complete insert did not survive regeneration via organogenesis, since this comprises a callus phase. Consequently, a reduction of barnase expression from the uninduced promoter was necessary. For this purpose, four different ORFs, which in another system (Ruan et al., 1996; Mize et al., 1998) inhibit translation of downstream ORFs
between 8- and 20-fold, were introduced upstream of the barnase ORF. The yield of plants containing the complete insert was slightly better in these transformation experiments. A total of 6 plants containing the barnase-ACMV promoter-barstar fragment with a sORF could be regenerated, but still the amount of plants with rearranged copies of the transformed plasmid was higher than expected. Probably, these plants would usually regenerate slower than plants containing a normal copy of the transformed plasmid and would not be detected in an experiment without toxic genes involved. Since in this case, a gene leading to the production of a toxin, the barnase, was used for transformation, plants containing rearranged copies of the barnase ORF might have an advantage and might regenerate faster and more successfully compared with plants with a complete barnase ORF. Furthermore, the variability of transgene expression, as demonstrated by the higher activity of Renilla luciferase (corresponding to barnase) as compared to firefly luciferase (corresponding to barstar) observed in line slr9 (see Chapter 2), could be another explanation for the low number of plants regenerated containing an intact copy of the transgene. If at one stage during regeneration more barnase than barstar is present in the cell, no plants will be able to regenerate from these cells.

The regeneration of plants containing an intact copy of the transgene, was slower when compared to wildtype regeneration, but all plants developed normally, except for those of line sbb17. The observed random pattern of brown spots spreading on older leaves of sbb17 seems to be due to barnase activity. As these spots first appear on older leaves, it could be speculated that barnase is slightly more stable than barstar and slowly accumulates until more barnase than barstar is present in the cell. The death of such a single cell might also affect surrounding cells either directly through the barnase or by initiating a hypersensitive response. It is also possible, that the relative level of barnase to barstar expression is not exactly the same in all the cells of a plant, that some produce slightly more barnase than others, leading to stress and consequently to a hypersensitive response. As these brown spots were not observed in any of the other transgenic lines, it can be assumed, that line sbb17 has the most fragile balance of barnase to barstar and that in all the other lines relatively less barnase is produced. That such a variation is likely to occur can be concluded from results obtained with transgenic plants containing the ACMV-luciferase construct (see Chapter 2).

The results of the replication assays with leaves of transgenic plants compared to wildtype leaves clearly show that replication of viral DNA is severely reduced in transgenic plants of all tested lines. The highest reduction of replication was seen for plants of line sORF+11 and sbb17, which does suggest that the balance between
barnase and barstar is actually more fragile in these plant lines than in the others containing sORFs. It cannot be expected that replication of viral DNA is completely abolished due to the barnase. As long as the barnase is activated before the virus can spread out of the infected cell, the plant would still be resistant even if some replication occurs. In order to find an optimum balance of a high and fast barnase production which the plant can still support even in older leaves, more transgenic plants need to be regenerated.

Since *Nicotiana tabacum* SR1 plants could not be agroinoculated with ACMV, even though replication in SR1 protoplasts was shown, it was not possible to test whether also the spreading of the virus would be reduced or inhibited. However, the observed resistance of plants containing the dianthin under the control of the ACMV promoter (Hong et al., 1996; Hong et al., 1997), strongly suggests that our approach could provide resistance against ACMV in the future.
Chapter 4

Cassava regeneration and transformation

4.1 Abstract

Transformation and regeneration of cassava (*Manihot esculanta* Crantz), an integral plant for food security in developing countries, using different systems has been published before. These protocols are all developed for two cassava model cultivars. Since cassava is grown as many different land races, it is crucial to adapt these methods to various cultivars. The adaptability of transformation systems based on *Agrobacterium* or particle bombardment to an African cultivar and the changes needed in the published protocols for regeneration of transgenic plants of this cultivar were assessed in this work.

4.2 Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial shrub, belonging to the family Euphorbiaceae. It is one of the most important sources of calories produced within the tropics (Cock, 1982; Puonti-Kaerlas, 1998) and provides food for over 500 million people. Cassava roots can be processed into a wide variety of products for food and industrial uses and since most of the processing can be done locally, it provides rural jobs and income (Henry et al., 1995). Cassava is grown because of its robustness and its reliability as a superior producer of carbohydrates even on poor soil and for its ability to survive seasonal drought and other adverse environmental conditions (Thro et al., 1995). Up to 80 t/ha roots can be produced under optimal conditions in a 12 month growing season, but yields are severely reduced by insect pests and diseases,
that cause up to 70 - 80% losses locally (Roca et al., 1992). During the last decade the interest in cassava breeding has grown and it has been recognized that biotechnology might be a useful tool for genetic improvement of cassava; especially since traditional breeding is very difficult. High heterozygosity, irregular flowering, low seed set and variable germination rates constrain the crossing of different cassava cultivars to combine desirable traits. Genetically engineered plants could complement these efforts and contribute to a sustainable agriculture and an improved diet in the third world countries.

Gene transfer to plant cells can be performed by two different systems: direct gene transfer or *Agrobacterium*-mediated gene transfer. Direct gene transfer methods, such as microinjection, PEG treatment, calcium-phosphate precipitation or electroporation, mostly need specific protocols to regenerate fertile plants from protoplasts. On the other hand, direct gene transfer through bombarding small DNA-coated particles to intact cells can be used to transform organized tissues (Klein et al., 1988). One of the devices that were developed for biolistic transformation is the Particle Inflow Gun, which is inexpensive and simple to use (Finer et al., 1992). Both methods have been used to transform cassava: cotyledons from somatic embryos were transformed via *Agrobacterium* and particle bombardment (Li et al., 1996; Legris et al., 1998) and embryogenic suspensions were transformed via *Agrobacterium* and particle bombardment (Schöpke et al., 1996; Raemakers et al., 1996; González et al., 1998).

As cassava is grown as many different land races, it is crucial that these regeneration and transformation systems can be applied to a wide range of cultivars. The transferability and adaptability to an African cassava cultivar were assessed in order to engineer African cassava mosaic virus resistant cassava.
4.3 Materials and methods

4.3.1 Plant material

In vitro culture of cassava (Manihot esculenta Crantz)

Cassava cultivars TMS 60444 (kindly provided by IITA) and MCol22 (kindly provided by CIAT) were maintained as shoot cultures in Magenta-vessels on basic medium BMS (MS salts and vitamins (Murashige and Skoog, 1962) with 2% sucrose, supplemented with 2 μM CuSO₄ (Schöpke et al., 1993) and solidified with 0.7% agar, pH 5.8). The cultures were kept at 25 °C at an 18 h photoperiod and subcultured every 8 weeks, transferring the shoot apexes to fresh medium.

Suspension cultures

Embryogenic suspension cultures were established from friable embryogenic calli of cultivar TMS 60444. The suspensions were grown axenically in liquid SH medium (SH salts with MS vitamins, 6% sucrose and 12 mg/l picloram, pH 5.8 (Schenk and Hildebrandt, 1972; Taylor et al., 1996)) on a rotary shaker (80 rpm) under continuous low light in a growth chamber (Infors Incubator HT04). Suspensions were subcultured once to twice a week depending on the desired growth rate. Every four weeks the suspensions were filtered through a 1 mm² mesh sieve and larger particles were discarded. For maturation, the suspensions were transferred to solid maturation medium (BMS with 4% sucrose and supplemented with 1 mg/l picloram) and subcultured every two weeks as described (Raemakers et al., 1996). Variations of maturation medium consisted of BMS supplemented with 0.5 mg/l picloram, 2 mg/l picloram 1 mg/l NAA or no picloram. Mature embryos were transferred to germination medium (BMS supplemented with 1 mg/l BA) for shoot development.

Regeneration via organogenesis

Cassava meristems were excised from shoot cultures and transferred to BMS containing 12mg/l picloram (Taylor et al., 1993) to induce embryo development. To remove faster growing callus, somatic embryos were subcultured every 4 weeks, thereby establishing a cyclic somatic embryo system. Mature somatic embryos were harvested and transferred to maturation medium (BMS supplemented with 0.1 mg/l BA). Green cotyledons from maturing embryos were cut into pieces of 1 - 3 mm² and transferred to organogenesis medium (BMS supplemented with 1 mg/l BA and 0.5 mg/l IBA). After 20 days of induction, shoots or organogenic structures could be detached and transferred to elongation medium (BMS supplemented with 0.4
mg/l BA). Elongated shoots were moved to BMS for rooting. Additional shoots could be harvested until up to three months after induction. In order to enhance regeneration, various organogenesis and elongation media were tested. Organogenesis medium with different auxins (0.1 mg/l IBA, 0.25 mg/l IBA, 0.1 mg/l NAA or 0.25 mg/l NAA), with different concentrations of BA and IBA (1.5 mg/l BA with 1 mg/l IBA, 1 mg/l BA with 0.8 mg/l IBA, 0.5 mg/l BA with 0.1 mg/l IBA and 0.5 mg/l GA₃ or 1 mg/l BA without IBA) and organogenesis medium prepared with B5 salts (Gamborg et al., 1968) were tested in different experiments. Furthermore, an elongation medium with additional GA₃ at 0.1 mg/l was tested. Recent results arising during the course of these experiments showed that AgNO₃ had a positive effect on organogenesis of cassava (P. Zhang, pers. comm.), therefore, AgNO₃ was added routinely to organogenesis medium at a concentration of 4 mg/l in later experiments.

4.3.2 Bacterial strains and constructs

E. coli strain XL1-Blue was purchased from Stratagene (La Jolla, CA, USA). A. tumefaciens strain GV3101, containing helper plasmid pGV2260 (Deblaere et al., 1985) was kindly provided by B. Finnegan, Zurich.

All DNA manipulations were performed according to standard procedures (Maniatis et al., 1982). Nucleotide numbering of the ACMV genome refers to pJS092 and pJS094 (Stanley and Davies, 1985). DNA A and names of the open reading frames are according to suggested nomenclature (Davies and Stanley, 1989), unless the gene function is known. In this case the name of the gene product is indicated.

The bidirectional ACMV promoter was produced by PCR amplification of ACMV DNA A using primers ACMVe (GGAA -2755- GCTTTTTGACCAAGTCAATT- GG -2776) and ACMVb (300-GTGGTACCCACTATTGCGCACTAGC -267). The ACMV promoter was introduced as a KpnI/MunI fragment between the two tobacco scaffold attachment regions (SAR) (Breyne et al., 1992) in the backbone of pGluChi (Bliffeld et al., 1999). The barstar ORF (Hartley, 1988) was introduced as an Ncol/PstI fragment under the control of the AC1 promoter, while the barnase ORF (Hartley, 1988) (both kindly provided by J. Füttener, Zürich) was introduced as a blunt-end fragment into the blunt SphI site under the control of the AV1 promoter. Both ORFs were introduced together with a 35S terminator. The hygromycin resistance cassette was cloned as a blunt-end fragment downstream of the barstar gene, resulting in plasmid pACMVbb (see also Fig. 4.1). Correct cloning of inserts into the vector was confirmed by restriction digests and sequencing.

In order to transform plants with Agrobacterium, the barnase construct was introduced into pNC1 as a complete I-SceI-fragment. pNC1 is a pCambia1300 backbone
CHAPTER 4. CASSAVA REGENERATION AND TRANSFORMATION

Figure 4.1: Schematic representation of barnase expression cassette. Barnase expression cassette showing the position of the cloned ACMV DNA A promoter between the barnase and the barstar ORFs. The barnase ORF contains the intron from rice tungro bacilliform virus; both ORFs carry a 35S terminator (35S). An hpt ORF under the control of a 35S promoter (35SP), with a nos terminator (nos1) is included as a selectable marker. The whole expression cassette is flanked by scaffold attachment regions (SAR). The bar over the map represents the fragment amplified by PCR.

The resulting plasmid, pNCbb, was then electroporated (Mattanovich et al., 1989) into GV3101 (pGV2260) resulting in strain GVbarnase for agroinoculation of seedlings.

A defective interfering (DI) DNA (Stanley et al., 1990; Frischmuth and Stanley, 1991), kindly provided by T. Frischmuth, Stuttgart, was cloned together with a hygromycin resistance gene under the control of a 35S promoter (construct pNP1). The hygromycin resistance cassette was introduced as a SalI/BamHI fragment into the XhoI and BamHI sites of the DI DNA construct. For construct pDIluc, the same DI DNA was combined as a SalI fragment with a 35S-luciferase cassette as a HindIII/AatII fragment in a pSP72 vector (purchased from Promega, Catalys AG, Switzerland).

4.3.3 Transformation

Particle bombardment

Cotyledon explants were harvested from secondary embryos and transferred to transformation medium (BMS with 10% sucrose) 18 - 24 h prior to particle bombardment. Embryogenic clusters were collected from suspension cultures and spread on sterile filter papers on transformation medium 18 - 24 h before transformation. The particle inflow gun used is a microprojectile accelerator (Finer et al., 1992), which is equipped with a 2-way solenoid valve Lucifer E121K14 instead of the described one (ASCO, Flotham Park, NJ, Red Hat II). Commercially available gold-particles (Aldrich or BioRad) were coated with DNA using a protocol modified from EMBO Advanced Laboratory Course (Potrykus et al., 1993). Briefly, 2 μl DNA (0.1 μg/μl) were added into a tube containing 25 μl of gold-particle suspension (50 μg/μl) and DNA was
precipitated on particles using CaCl₂, spermidine and ethanol. The DNA-particle solution was vortexed shortly before loading 4.5 µl per shot. For bombardment, helium pressure was kept constant at 5 bar, the target plate was positioned at a distance of 15 cm and the 500 µm mesh stopping grid at a distance of 10 cm. Some parameters for transformation of cassava cotyledons have been partially optimized by monitoring of transient GUS expression (Legris et al., 1998). The bombarded cotyledons or suspensions were transferred to organogenesis or SH medium respectively 4 - 26 h after shooting and hygromycin, at a concentration of 15 or 17.5 mg/l, was added 3 days after bombardment of the cotyledons. Emerging shoots could be transferred to BMS between 20 days and 3 months after transformation.

Luciferase selection of transformed suspensions was performed using a slow-scan CCD camera (CCD 3200 LN/C, Astrocam Ltd, Cambridge, UK) that detects photons, which are emitted due to the turnover of the luciferin substrate by the luciferase. The luciferin substrate mixture (liquid BMS supplemented with 1 mM click beetle luciferin (Promega) and 1mM ATP (Kost et al., 1995)) was applied immediately before taking images on suspensions that were spread as a thin layer on solid SH medium. Reference images, to localize the emitted light from the explants, were taken by exposing the chip for 100 msec to reflected light. For bioluminescence images, the time with an open diaphragm, was 10 min. Luciferase detection for selection was performed every 3 to 4 weeks. Luciferase positive embryonic clusters were isolated and either transferred to fresh SH medium for further multiplication or transferred to maturation medium and subcultured every 2 weeks.

**Agrobacterium-mediated transformation**

Strain GVbarnase was grown for 2 days in liquid AB medium (Chilton et al., 1974) containing the appropriate antibiotics at 28 °C on a rotary shaker (280 rpm). Before cocultivation, the bacteria were diluted to OD₅₆₀ 0.5 and preinduced for 5 hours in liquid BMS supplemented with 200 mM acetosyringone, pH 5.2. Cotyledon explants from secondary embryos were inoculated with Agrobacterium for 30 min, transferred to cocultivation medium (BMS supplemented with 100 mM acetosyringone, 1 mg/l BA and 0.1 mg/l IBA) and cocultured for 3 days. Explants were washed with liquid BMS, blotted dry on filter paper and transferred to organogenesis medium supplemented with 500 mg/l carbenicillin and 10.5 mg/l hygromycin. After 20 days the first developing shoots were transferred to BMS.
4.3.4 Molecular analysis of resistant shoots

Total DNA from regenerated shoots and a wildtype control was extracted using the NucleonPhytopure kit (Amersham Life Science, England) as described by the manufacturer. PCR reactions (Mullis and Faloona, 1987) using 1 μg of plant DNA, were performed in a final volume of 50 μl using 5 units of Taq polymerase (QIAGEN, Hilden, Germany). Primers were added to a final concentration of 1 μM each and dNTPs (Boehringer Mannheim, Germany) were used at a concentration of 250 μM. PCR cycles consisted of a 1 min denaturation period at 95 °C, a 1 min annealing period at around 60 °C (depending on GC content and length of primer) and an elongation period of 1 min at 72 °C. Amplified products were separated by electrophoresis through an agarose gel. Primers were designed to amplify a 600 bp fragment of the barnase ORF and of part of the ACMV promoter (ACMVF (5'-CCACTATATACTTACAGGCC-3') and barnaseF (5'-CATGTTCGTCCGCTTTTGCC-3')) and a 550 bp fragment of the hygromycin resistance cassette (35Shpt5 (5'-GAAAGGAAGGTGGCTCC-3') and hpt3 (5'-AATAGGTCAGGGCTCTCGC-3')). As internal control primers Q3 (5'-TGACGAGAATCATCAATGT-3') and Q4 (5'-TACAATGGGCTTGGTATGC-3'; kindly provided by S.Bohl, Zürich) were used to amplify a 380 bp fragment of the branching enzyme (Kallak et al., 1997).
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4.4 Results

4.4.1 TMS60444 transformation and regeneration via organogenesis

Somatic embryos were subcultured every four weeks either to fresh embryo induction medium or to maturation medium. Cotyledon explants were harvested for particle bombardment after 10 to 14 days or as soon as cotyledons were light green and around 5 - 10 mm². Three days after bombardment with pACMVbb or pNP1, selection using hygromycin was started at a concentration of 15 mg/l. The first organogenic structures could be seen after 4 to 6 weeks on organogenesis medium in about 90% of the explants, although these structures looked slightly different from organogenic structures of MCol22 (see Fig.[4.2]), resembling primary embryos emerging from a green, elongated structure. When these structures were transferred to elongation medium, no shoots emerged and the embryo-like bulbs eventually dried out. Subculturing to fresh organogenesis medium also had the same effect. None of the tested organogenesis media induced production of any structures similar to organogenesis of MCol22, neither did the elongation medium supplemented with GA₃, improve regeneration. A summary of some of the tested media is shown in table 4.1. Organogenesis medium with only 0.5 mg/l BA (instead of 1 mg/l) or without IBA produced roots on 2% of the explants while higher concentrations of BA (2 mg/l) produced mostly callus and few organogenic structures. Root formation was inhibited if explants were kept in the dark but callus formation was further enhanced. The most explants with organogenic structures (85%) were observed on a medium containing 1.5 mg/l BA with 0.5 mg/l IBA cultured in the light and on the standard medium (95%), but no shoots could be regenerated. In addition, experiments with organogenesis in the dark and the different transformation conditions tested (variable times of pre- and postplasmolysis) did not have any positive effect on the regeneration of shoots. At least 50 explants were used for each individual treatment and out of a total of around 8000 bombarded explants only 4 lines could be regenerated and rooted. All four lines were regenerated using the standard protocol, with two lines bombarded with the barnase construct and two with the DI construct. Neither the presence of the barnase gene/DI DNA, nor the hygromycin resistance gene could be confirmed by PCR analysis. The internal control, part of the branching enzyme, was correctly amplified for all four plants.
4.4.2 MCol22 transformation and regeneration via organogenesis

As regeneration of TMS60444 proved very difficult, it was decided to use cultivar MCol22 for further experiments. Secondary somatic embryos of MCol22 (kindly provided by S. Phansiri, Bangkok) were germinated and bombarded using FIG or in one experiment (300 explants) *Agrobacterium*. Since non-transgenic plants regenerated under selection, a higher concentration of hygromycin was used for selection (17.5 mg/l) and also AgNO₃ was added routinely to the organogenesis medium. Organogenic structures could be detected as soon as 3 weeks after bombardment and shoots could be harvested until up to 3 months later. Out of 6200 explants bombarded with pACMVbb, 7 lines could be regenerated and of 2700 explants bombarded with pNP1, 4 lines could be regenerated. No shoots could be regenerated from the *Agrobacterium*-mediated transformation experiments. Each organogenic structure (origin of one plant line) produced more than one shoot (see Fig.[4.4]). The 11 regenerating lines produced about 50 shoots in total of which about 90% could be rooted in BMS. Neither the barnase ORF nor the DI DNA, nor the hygromycin resistance gene could be detected in any of the lines by PCR analysis.
CHAPTER 4. CASSAVA REGENERATION AND TRANSFORMATION

Table 4.1:

<table>
<thead>
<tr>
<th>Hormone conc. (mg/l)</th>
<th>% of explants with org. structure</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BA</td>
</tr>
<tr>
<td>1 0.5</td>
<td>95</td>
</tr>
<tr>
<td>0.5 0.5</td>
<td>50 a</td>
</tr>
<tr>
<td>1.5 0.5</td>
<td>85</td>
</tr>
<tr>
<td>2 0.5</td>
<td>30</td>
</tr>
<tr>
<td>1 0</td>
<td>10</td>
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*a*additional root formation

The expected part of the branching enzyme was correctly amplified in all lines as an internal control. An example of a PCR with some of the shoots bombarded with the barnase construct is shown in Fig.[4.3].

4.4.3 Transformation and regeneration of suspension cultures

Suspension cultures of cultivar TMS60444 were established from friable embryogenic callus (FEC) and maintained as described (Taylor et al., 1996). Freshly filtered suspensions were used for transformation, spreading a thin layer of suspension on a filter paper of 2 cm diameter on transformation medium. All suspensions were bombarded using the particle inflow gun and the construct pDIlluc. After transformation, the suspension cells of one experiment (10-20 dishes) were combined and transferred back to liquid SH medium. Three to four weeks after bombardment, suspensions were spread on solid SH medium and analyzed for luciferase activity. A 1 cm² region of suspension around the luciferase positive spot was isolated and cultured individually in liquid SH medium. Only after the second round of selection these luciferase positive foci were transferred to solid maturation medium. All transformed suspensions (200 dishes in 10 experiments) had single luciferase positive spots 3 - 4 weeks after transformation (around 10 - 20 spots per experiment), however, about 50% of these were not positive when tested for the second time. Furthermore, around 20 - 30% of the isolated luciferase positive suspensions turned white and stopped dividing after the luciferase assay. Therefore, in later experiments, transformed suspensions were tested for luciferase activity only 2 months after bombardment, before transfer to maturation medium. In these cases only very few luciferase positive spots (1 -
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Figure 4.3: Gel electrophoresis of PCR analysis of cassava transformed with pACMVbb. No amplified product of the barnase could be detected in any of the tested shoots. As an internal control, a 380 bp fragment of the branching enzyme was amplified for all shoots. 10 ng of pACMVbb were used as a positive control for the barnase PCR; sterile water served as a negative control.

2 per experiment) could be detected. Four experiments still had luciferase positive cell clusters 4 months after transformation (2 months on maturation medium), but none of these could be regenerated. Maturation of embryos was achieved only in one experiment (luc5) but, when transferred to germination medium, these embryos did not develop further. No improvement of maturation could be observed with the various tested hormone concentrations in the medium. Only in maturation medium containing 1 mg/l NAA two embryos with green cotyledons were found, but after transfer to germination medium both of these turned white and did not develop further.
Figure 4.4: Regeneration of multiple shoots from organogenic structure of MCol22 after 3 months on elongation medium.
4.5 Discussion

Production of transgenic cassava plants has been reported using both *Agrobacterium* and particle bombardment (Li et al., 1996; Raemakers et al., 1996; González et al., 1998) and regeneration via organogenesis of cotyledon explants or via germination of embryogenic suspensions. Applying these transformation systems to different cultivars of cassava is important as cassava is grown as many land races and often exhibits low fertility. Therefore, crossing of new genes into local varieties might be difficult. The goal of this work was to study the applicability of the biolistic transformation system together with the regeneration via organogenesis to the African cultivar TMS60444.

The regeneration frequency of cassava cultivar TMS60444 was low under all the different conditions tested and only few shoots could be regenerated and rooted. This could be due to different reasons. Genotype dependent variations of regeneration have been reported before (Kartha, 1974; Kallak et al., 1997), therefore it is not surprising that a protocol established for organogenesis of MCol22 does not apply to the organogenesis of TMS60444. More profound adjustments would be needed, since our results demonstrate that minor changes in hormone concentrations of the standard media could not enhance regeneration. Preliminary results in our laboratory show a considerable effect of AgNO₃ on the regeneration of this cultivar (P. Zhang, pers. comm.), however, at the time of these experiments, these results were not available. The positive effect of AgNO₃ on organogenesis of oil seed *Brassica* species (Eapen and George, 1997) and on regeneration on a variety of plants (Songstad et al., 1988; Chi et al., 1990) has been reported before, although the actual mode of action has not yet been elucidated.

Regeneration of MCol22 proved to be more efficient, especially in combination with particle bombardment, which in fact enhanced the formation of organogenic structures. The wounding of cells by particle bombardment and the thereby triggered wound reaction could be responsible for this effect. Nevertheless, not all organogenic structures could be regenerated to shoots. The step between the first small shoot primordia and the complete shoot is the bottleneck in the regeneration via organogenesis. Therefore, optimization of the protocol should not only be concentrated on the organogenesis medium, but also on the elongation medium and on the timing of transfer of organogenic structures to elongation medium. Our results indicate that it is crucial to use only 10- to 14-day-old cotyledons for transformation, since older cotyledons form less organogenic structures and cannot be regenerated to shoots, and younger cotyledons are too small to be used.

Regenerated shoots of both TMS60444 and MCol22 were not transgenic, as
shown by PCR analysis. Transformation with barnase - even in combination with barstar - can be difficult (see Chapter 3; (Mariani et al., 1990)) due to its high toxicity, which leads to early loss of transformed cells. Also DI DNA, consisting of part of the ACMV DNA B, might have a negative effect on plant cells. As the presence of the hygromycin resistance gene could not be proven either, the regenerated shoots must have escaped selection. Selection with a higher concentration of hygromycin (17.5 mg/l) could not prevent the regeneration of untransformed shoots either, even though shoot regeneration could be efficiently suppressed in control explants. It appears that transformation via particle bombardment requires a tighter selection system, since recovery of non-transgenic plants was not a severe problem after Agrobacterium-mediated transformation (Li et al., 1996). This problem could be partly due to the enhanced regeneration observed after bombardment, or to the smaller sectors transformed by particle bombardment as compared to Agrobacterium-mediated transformation. As shoots regenerated via organogenesis do not originate from a single cell, it is less likely that a completely transgenic shoot primordia is formed by biolistic transformation and small transformed sectors could be lost after partial shoot formation due to the negative effect of the barnase gene. Since cassava is grown as many landraces, biolistic transformation, which is less genotype-dependent, may still be desirable to complement transformation using Agrobacterium. Further method development will be needed and selection will have to be optimized. A selection system using luciferase expression in addition to antibiotic selection for monitoring transgenic shoots has been proposed. Luciferase-based selection has the advantage that no non-transgenic shoots will be regenerated, without further enhancing stress due to high antibiotic concentrations.

A luciferase-based selection system was applied for the regeneration of transgenic plants from suspensions. Luciferase positive embryogenic clusters could still be observed up to four months after transformation, but also here regeneration to complete shoots proved to be difficult. Different factors may have influenced the regeneration frequency. One is the age of the suspension culture used, since younger suspension cultures seem to regenerate more easily and less somaclonal variation, due to a prolonged time in tissue culture, would be expected. Our experiments indicate a connection between suspension age and regeneration capacity (data not shown, P. Zhang, pers. comm.). Furthermore, the stress caused by luciferase expression, which requires ATP, might affect transformed cells negatively in comparison to untransformed cells. These would then grow faster and supersede the luciferase positive cells. Culturing smaller volumes of suspensions (i.e. transferring less untransformed cells after the luciferase assay) is not optimal, since a certain density
of cell volume in the liquid medium is required for the survival of the suspension. Regeneration has now been enhanced by using 1 mg/l NAA instead of picloram in the maturation medium and by transferring cotyledons of regenerated mature embryos to organogenesis medium, instead of germinating embryos directly at a lower frequency (P. Zhang, pers.comm.). This transformation system has been further improved by selecting with 50 mg/l hygromycin and transforming with Agrobacterium and is now applied routinely in our laboratory (P. Zhang, in prep.).
Chapter 5

General Discussion

Hunger and malnutrition are among the most severe problems in many areas of the world, particularly in Africa. At present, over 800 million people are starving every year while the world population increases by 85 million people (FAO, 1998). It has been estimated that food production has to be doubled during the next 25 years in order to keep up with the world population growth. As the land available for agriculture is declining due to urbanization, soil erosion and salinization (Brown and Kane, 1994), the future increase in food production must come from higher productivity and more efficient use of the existing resources under sustainable conditions.

Among the traits to engineer in cassava, resistance against African cassava mosaic virus (ACMV) has been listed as the highest priority in the Cassava Biotechnology Network (CBN, 1995), which was confirmed again at the CBN meeting in Uganda 1996. ACMV is the most devastating disease in cassava, leading to total crop failures locally and also threatening certain varieties with extinction. The benefits of virus resistant cassava can only be calculated approximately by using the figures of estimated losses, which e.g. in 1993 in Uganda reached US$ 180 million per year (Otim-Nape et al., 1994b). In case of subsistence farmers, at present severely threatened in their existence by acute food shortage, the cost in human lives cannot be estimated in dollars.

The goal of this thesis was to develop and evaluate a new ACMV resistance strategy in a model system and to assess the possibility of transferring this resistance to an African cassava cultivar. The resistance is based on a regulatable toxin/antitoxin system mimicking a hypersensitive reaction by using the regulated ACMV promoter in combination with the barnase, an RNase of Bacillus amyloliquefaciens, and its specific inhibitor, the barstar (Hartley, 1989).

The properties of ACMV promoter regulation were first studied transiently in tobacco and cassava protoplasts and in stably transformed tobacco as a model plant.
system using two different luciferases as reporter genes. The bidirectional promoter of African cassava mosaic virus is regulated by viral proteins during the infection cycle, permitting a discrimination between early and late genes. The following model has been proposed: Early after infection the complementary-sense gene products of Rep (replication associated protein), TrAP (transcription activator protein) and REn (replication enhancer protein) are produced. Rep then reduces its own transcription while REn enhances replication and TrAP activates virion-sense transcription. The late genes CP (coat protein) and NSP (movement protein) are produced and encapsidation and spread of viral DNA begins (Frischmuth, 1999). The results on promoter regulation presented in this thesis are essentially in agreement with and confirm the presented model, but in addition draw a more differentiated picture of ACMV promoter regulation. It could be shown that TrAP not only activates expression of the v-sense ORFs but also of c-sense ORFs (see Figs. [2.4] and [2.5]) and that activation of c-sense expression is inhibited by small amounts of Rep. Our results using the complete DNA A for cotransformation can confirm the aforementioned model: a downregulation of c-sense expression and a strong upregulation of v-sense expression.

Surprisingly, the regulation of the DNA B promoter in cassava proved to be the opposite from that of the DNA A promoter, with no significant effect on v-sense expression and an upregulation of c-sense expression. NSP has been shown to transport the DNA out of the nucleus (Sanderfoot et al., 1996) while the MPB forms endoplasmatic reticulum-derived tubules that extend through the cell wall to the next cell (Ward et al., 1997). In the case in which only NSP is transiently expressed in protoplasts, the protein can be observed solely in the nucleus, while, when cotransformed with MPB, it can be found also in the cytoplasm (Sanderfoot and Lazarowitz, 1996; Lazarowitz and Beachy, 1999). It can be speculated that the enhanced production of movement protein helps relocating the nuclear shuttle protein NSP with bound viral DNA to the cytoplasm, where it can be transferred to adjacent cells, thus spreading viral DNA.

On the other hand, the upregulation of v-sense expression is not as pronounced in tobacco cells, therefore, host factors, which are different in cassava and in tobacco, seem to have an influence on the promoter regulation. Similar observations of different results from infection of a model plant as compared to the natural host, are reported for Beet curly top virus infecting N. tabacum and B. vulgaris, respectively (Frischmuth et al., 1993a). This illustrates the limitation of model systems and emphasises the necessity of an assay system that actually reflects the natural situation. All transient assays reported here, were performed in protoplasts origi-
nating either from mesophyll cells, for tobacco, or from suspension cells, for cassava. This method is extremely convenient since it is simple, fast, and has the advantage that the cells used are quite uniform. Nevertheless, the behavior of a whole plant cannot be entirely inferred from the results obtained from protoplasts, which are single cells subjected to stress during protoplast isolation and transformation.

A logical step to follow up transient expression assays is the stable integration of reporter genes to study promoter regulation on the whole plant level. As it was technically impossible to produce transgenic cassava plants, we again relied on tobacco as a model plant, in spite of the limitations of this system. In experiments with tobacco plants containing the ACMV-luciferase constructs, several discrepancies with earlier transient expression studies could be found. First, the basal firefly luciferase expression in transgenic plants was higher than the expression of Renilla luciferase, while in transient expression assays in protoplasts this relation was reversed. These results seem very promising for our intended virus resistance strategy, since this would indicate, that expression of the barnase and barstar from the ACMV promoter could be tolerated in transgenic plants, as more barstar than barnase would be produced in a non-infected cell. Second, in protoplasts of transgenic plants, the activation of luciferase expression by TrAP alone could not be observed anymore. It appears as if the regulation of the integrated ACMV promoter did not correspond to that of the viral promoter during a natural infection. For our purpose of developing a virus resistance strategy using the integrated, regulatable ACMV promoter, however, it was necessary to show that DNA A can still regulate an integrated promoter. This was confirmed by our results. Additionally, our results from different plant lines illustrate the necessity for the production of many different transgenic lines with the same construct, since basal levels of reporter gene expression and subsequent upregulation varied strongly between separate lines. The variation in expression could be due to different integration sites in the genome, but also to the number of transgenes integrated. Judging from our results, further testing of the regulation of the ACMV promoter in transgenic cassava plants would be required, although results from cassava protoplasts and from transgenic tobacco plants indicate that the ACMV promoter can still be regulated, even when integrated in the plant genome. Since the cassava transformation system is not routine enough yet, production of large numbers of transgenic plants with the luciferase construct was not feasible during the course of this thesis.

Another important factor to consider is that the DNA structure of the integrated promoter is different from the one of an infecting virus. That this aspect has to be considered could be shown by experiments comparing linear and supercoiled input.
DNA. We could show that when linear DNA was used for cotransformation TrAP did not function as an activator anymore and only when the complete DNA A was used for transformation, a slight increase of v-sense expression could be found. The structure and context of the DNA is important in promoter regulation and transcription (Gutierrez et al., 1995; Fry and Farnham, 1999; Muro-Pastor et al., 1999). It might be possible, that a certain bending of the DNA is needed in order to bind TrAP. On the other hand, when the complete DNA A is expressed, other proteins, like the Rep or the REn, might help to remodel the promoter DNA, such that TrAP can activate transcription.

It is shown in transient protoplast assays, that Rep downregulates both v- and c-sense transcription, while TrAP enhances transcription from both sides. Only when both proteins are present, after cotransformation with the DNA A, a side specific regulation of transcription can be observed. This can be explained by the fact that there is either an interaction between Rep and TrAP or that also the REn is necessary for viral promoter regulation. The fine tuning of this system seems to be very delicate, as results gained by cotransforming various ratios of the Rep and TrAP constructs with the pACMVs showed a high variation. Furthermore, the effect of REn, which might still play a role in the regulation process, was not systematically studied in these experiments. It could be speculated that by enhancing replication, the transcription rate might be altered due to a shift in the availability of certain viral or host factors.

The results on promoter regulation presented in this thesis show that a viral promoter integrated in the plant genome can be regulated by the ACMV DNA A. This may indicate that an infecting virus could achieve an analogous result. Furthermore, it could be shown that in transgenic plants, the basal level of c-sense transcription is higher than v-sense transcription, indicating, that in plants transformed with pACMVbb, more barstar than barnase would be produced, thereby neutralizing the toxic effect of the barnase. The presented results imply that the ACMV promoter is usable in the intended strategy of mimicking a hypersensitive reaction.

Obviously, many parameters of the presented virus resistance strategy cannot be simulated using reporter genes. Therefore, tobacco was chosen for pACMVbb transformation, although even this proved to be problematical. Of the first 8 transformation experiments, only one plant containing the complete insert could be regenerated. Possibly, in most lines the barnase was expressed at higher levels than the barstar even from an uninduced promoter and, therefore, only those with partial copies of the barnase could be regenerated. Another possible explanation is the different regulation or non-existent regulation of the ACMV promoter at the callus
level. For tomato golden mosaic virus it has been shown that repression of v-sense transcription is not maintained in unorganized tissue (Sunter and Bisaro, 1997). As transgenic tobacco plants are regenerated through a callus phase with subsequent organogenesis, it could be that explants containing an intact copy of the barnase are lost at this stage due to uncontrolled production of barnase. The further downregulation of barnase translation, using different short ORFs upstream of the barnase ORF, enabled the regeneration of further transgenic plants. Nonetheless, regeneration frequencies were still lower than usual, and many lines containing incomplete barnase ORFs could be detected by Southern blot (data not shown).

Regenerated plants, containing the complete gene fragment, were tested in a transient ACMA-* replication assay by shooting autonomously replicatable ACMV DNA A into leaf pieces. The amount of replicated viral DNA in shot wildtype leaves and in shot transgenic leaves could then be compared. In all tested transgenic lines, viral replication was considerably reduced, showing that, due to the regulation of the ACMV promoter, barnase production is in fact enhanced. Beside presenting the first indications that this system could generate virus resistant plants, this assay can also be used to identify transgenic plants which are silenced or plants in which the barnase expression cannot be upregulated sufficiently. Results from replication assays, however, do not give any indication on the kinetics of gene regulation leading to barnase production. It cannot be concluded that plants exhibiting reduced viral replication will actually be virus resistant in the field. For our purpose it is important to confine the virus to as few cells as possible and cause local cell death before viral replication and viral movement takes place. Otherwise, cell death due to barnase expression will spread along the leaf together with the virus. It is possible to specifically destroy infected cells using the ACMA* promoter before the virus can spread to adjacent cells, as has been shown before with dianthin (Hong et al., 1996; Hong et al., 1997). Our system is slightly more sophisticated, however: the barnase first has to overcome its antitoxin barstar, which already is present in the cells at a low concentration. Whether the retardation of the barnase due to the presence of barstar is crucial cannot be deduced from results obtained with reporter genes or the replication assay. Although, considering the high variation of responses in the different plant lines containing the ACMV-luciferase construct, it can be expected that some of the generated ACMV-barnase plants will be able to produce enough barnase to overcome the antitoxin and trigger cell death before the virus is able to replicate and spread.

It is unclear, why N. tabacum SR1 plants could not be infected with ACMV. N. tabacum cvs. Samsun and Xanthi plants have been previously used in infection
studies (Frischmuth et al., 1993b), nevertheless, no viral DNA could be detected in agroinoculated SR1 tobacco plants 2 - 3 weeks after transformation. As we have shown in the replication assays that replication of ACMV DNA A does take place in SR1 plants, it has to be assumed, that the movement of the virus is impaired in these plants. Whether the inability of systemic movement is due to MPB or NSP can only be speculated since both movement proteins have to interact with components of the host cell. Also the infection with another geminivirus did not provide any conclusive results. Possibly, SiGMV, a virus from the New World, was not closely related enough to regulate the ACMV promoter.

A next logical step in testing virus resistant plants, would be the infection through the natural vector, the B. tabaci. The results from these experiments could still be slightly different than after agroinoculation, since then the virus is inoculated directly in the leaf mesophyll cells and not through the phloem. Furthermore, the DNA introduced through agroinoculation has to excise itself from the plant genome, resulting directly in a double-stranded DNA, while naturally introduced viral DNA is single-stranded and transcription cannot occur directly. Replicated ACMV DNA can only be found 2 - 3 days after inoculation, while luciferase expression driven by the ACMV promoter can be measured already after 24 hours. It thus appears that gene transcription and subsequent promoter regulation occur before viral replication. These results, however, were obtained in transient assays with double-stranded viral DNA, and it is not clear whether the single-stranded form of naturally occurring viral DNA would behave differently. Moreover, whiteflies can feed in more than one site on a cassava leaf and thereby deposit ACMV in various cells across the leaf. Depending on how far the virus can spread and how fast it can replicate before local cell death due to the barnase can prevent further spreading and replication, but also depending on how far the induced local cell death spreads due to barnase to adjacent cells, damage might be considerable, even if systemic infection is prevented. Once more, it is expected, that under a large number of transgenic lines, the ideal tuning of the toxin/anti-toxin system can be found.

Provided that the transgenic plants produced during the course of this thesis are virus resistant, the next question is how easily this resistance could be overcome in the field. The ACMV promoter used in this strategy is conserved between the DNA A and DNA B. In order to inactivate the integrated promoter between the barnase and barstar, a mutation would need to take place in the promoter of both genomic components. Moreover mutations in both Rep and TrAP would then be necessary to regulate this new, mutated promoter but not the original promoter. The likelihood for this is statistically minimal. Another possibility for the virus to
overcome this resistance would be to accelerate replication and spread to adjacent cells before the barnase can overcome the barstar and cause local cell death. It can be assumed, that these faster viruses would spread through the leaf, initiating cell death in infected cells on their way and thereby destroying the plant before they can be spread to other plants through feeding whiteflies, thus becoming extinct in a fast manner.

As mentioned before, it is necessary to generate many different transgenic ACMV-barnase lines because the variation of transgene expression and viral transgene regulation between different lines is high. The cassava transformation system is still being improved and transgenic plants cannot be produced routinely yet. Given that the regeneration frequency of ACMV-barnase tobaccos containing an intact barnase ORF was very low (around 10% of all regenerated plants), it cannot be expected that transgenic cassava plants containing this construct can be produced at this stage. Not only would the necessary tissue culture work exceed our limits, also the molecular analysis would be labor-intensive for cassava, as long as selection is not improved. At present, too many non-transgenic plants can survive the selection scheme. These constraints in cassava transformation and regeneration should be overcome in the near future. The transformation system used now, has been improved, and more transgenic plants are being produced in our laboratory. Selection is tighter and at the same time the regeneration frequency could be improved. Therefore, it should be possible to generate virus resistant cassava employing the promising strategy presented in this thesis in the near future. These plants should then be tested not only by agroinoculation of the virus, but also by exposure to the whitefly vector and later, in the field. In case the \textit{in vitro} results reported in this thesis can be matched in the field, the same construct can either be introduced directly or through traditional breeding into other local varieties. As soon as other mechanisms of ACMV resistance are introduced in cassava, these different transgenes could be pyramided, resulting in broader range of resistance and in a more reliable resistance also under high infection pressure in the field, thereby contributing to a future sustainable cassava cultivation and to food security in Africa.
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