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„Analysis of the genetic stability of a GMO using real-time PCR and High Resolution Melting analysis“

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List of abbreviations

bp	base pair
C.	Confidence
CEN	European Committee for Standardization
CP4	A strain of *Agrobacterium tumefaciens*
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
EC	European Commission
e.g.	exempli gratia
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERA	Environmental Risk Assessment
EU	European Union
Fig.	Figure
GM	Genetically Modified
GMO	Genetically Modified Organism
HRM	High Resolution Melting
JRC	Joint Research Centre
Kb	kilo bases
nm	nanometre
No.	Numero
NTC	no template control
OECD	Organisation for Economic Co-Operation and Development
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rpm	rounds per minute
RR	Roundup Ready
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism

1. Introduction and Objectives

Since the introduction of genetically modified organisms (GMOs) in the European Union (EU) in 1997, the use of GMOs has drastically expanded. For the official control and authorization of food, feed and seeds an increasing number of GMO events have to be identified and quantified. In addition to the detection of GMOs it is a challenge to investigate the genetic stability of genetically modified (GM) plants [Hernandez, et al. 2005].

According to the European Food Safety Authority (EFSA) Guidance document and EU Directive 2001/18/EC, it is necessary that the introduced deoxyribonucleic acid (DNA) construct is stable and no changes in the DNA construct should occur [EFSA 2006, EU 2001]. Even small changes of the construct are undesirable because they may lead to unintended changes in the plant, its properties or ingredients, as well as changes to its morphology [Moch 2006]. In addition, incorrect quantification results may be caused by genetic differences in in the analysed samples [Broothaerts, et al. 2008]. However, according to the EFSA Guidance document and EU Directive 2001/18/EC, for the authorization of GMOs, GMO events have to be analysed for DNA rearrangements. No DNA instabilities should occur [EU 2001, EFSA 2006]. In praxis, for each GMO event only few samples are analysed by Southern Blots. Southern Blots are not suitable for the detection of Single Nucleotide polymorphism (SNP) - only DNA changes to several nucleotides can be detected. Thus, by the regulatory control no small DNA changes can be identified.

In this study, a high number of individual transgenic maize grains was analysed using methods that allow the detection of DNA changes consisting of just a few nucleotides. Real-time Polymerase Chain Reaction (PCR) and High Resolution Melting (HRM) analysis were used to investigate genetic stability. Finally the results were checked by Sanger sequencing. The sequencing results verified the presence of different SNPs as well as a larger deletion in the transgenic maize.

2. Literature overview

2.1. Legal background

2.1.1. EU Directive 2001/18/EC

EU Directive 2001/18/EC regulates the deliberate release of GMOs into the environment. According to that directive, the market release of GMOs requires a notification to the national authority of the Member State where the release is to occur. Next to other information, the notification must contain an environmental risk assessment (ERA). The competent national authority has to submit a report of the data to the European Commission and to the relevant authorities of the affected Member States. Then, an assessment takes place. Only after approval by the national authority is the deliberate release of GMOs permitted. The consent must be in agreement with the European Commission and the other Member States. Otherwise, objections are discussed in a certain period before a decision is made. Approvals are valid throughout the EU and for a maximum of 10 years [EU 2001].

The ERA must correspond to the principles described in Annex II of the EU Directive 2001/18/EC. Further to this, it must contain basic information listed in Annex III of the directive. It also has to be performed on a case-by-case basis. The aim is to identify and evaluate possible adverse effects of GMOs on human health or the environment. Such effects may occur directly or indirectly through different mechanisms such as the genetic stability of a plant. All relevant listed mechanisms which can lead to adverse effects should be evaluated in detail. Therefore, the verification of the genetic stability as well as influential factors are a necessary part of the ERA. The genetic stability of the insert is listed as basic requirement in Annex III [EU 2001].

According to EU Directive 2001/18/EC, after placing a GMO on the EU market, a post monitoring plan is necessary to uncover any adverse effects to humans, animals or the environment. The Regulation 1829/2003/EC on GM food and feed includes the obligation for applicants to implement an adequate GMO monitoring plan for environmental monitoring according to Annex VII of the Directive

2001/18/EC. According to the extent of the market release, adequate can mean concise or more detailed monitoring plans. The Council Decision 2002/811/EC describes in detail the structure of the necessary monitoring plan [EFSA 2010].

The GMO monitoring is based on the results of the pre-release ERA. Generally, it is divided into case-specific monitoring and general surveillance. The post market monitoring plan is a necessary part of the notification to the relevant member states [Environment Agency 2011]. The post market monitoring does not substitute the pre-market risk assessment. The purpose of post market monitoring is to increase the level of protection by increasing the probability of uncovering adverse effects [EFSA 2011]. The requirements for environmental release of GMOs for market placement are shown in figure (fig.) 1.

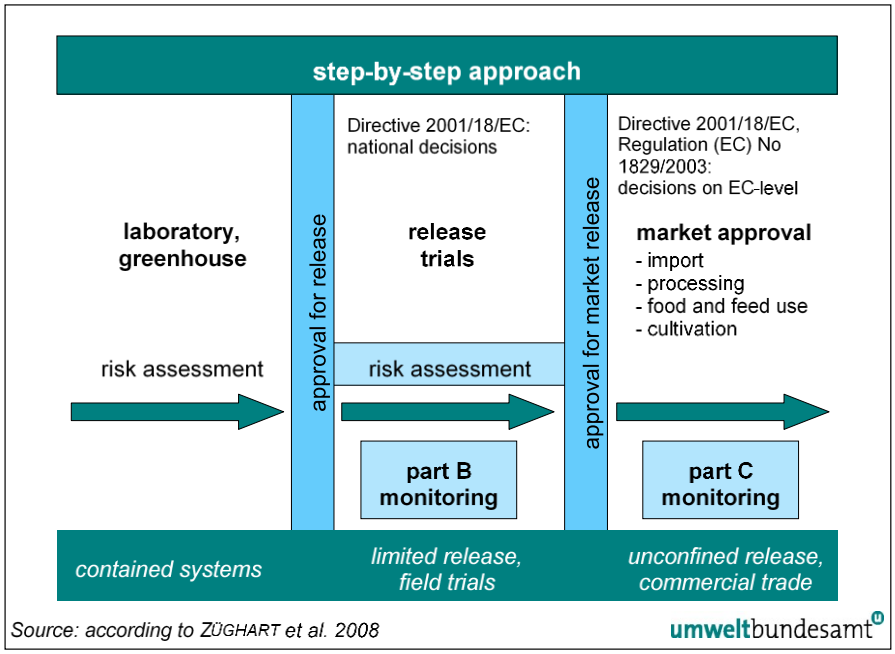

Fig. 1: Overview of the process of the environmental release of GMOs with regard to ERA and monitoring [according to Environment Agency 2011]

The action “part B monitoring” outlined in fig. 1 is part of the ERA and the obtained results influence “part C monitoring” which is synonymous with the post market monitoring. The “part B monitoring” data can be helpful to determine adequate parameters for the “part C monitoring” [Environmental Agency 2011].

“Part C monitoring” is not always necessary, e.g. MON810 needs no monitoring [EFSA 2009].

As the post market monitoring is individual for every event and, additionally, depends on the results of the ERA, genetic stability is not a mandatory parameter in post market monitoring.

2.2. Genetic stability

The genetic stability of the GMO is part of the molecular characterization in the risk assessment of GM plants [EFSA 2011]. The transgene construct of a GM plant must be stable. Every applicant must demonstrate the genetic stability of the transgenic locus before the product can released into the market. Furthermore, in the case of a stacked event the integrity of the transgenes must be retained. According to the “Scientific Opinion of the EFSA Panel on GMOs: Guidance for risk assessment of food and feed from GM plants”, for single events five generations or vegetative cycles are sufficient for the proof of genetic stability [EFSA 2011]. According to Aguilera et al. (2008) the genetic stability should be valid for the whole lifespan of a product [Aguilera, et al. 2008]. But in the performed studies regarding ERA little importance is ascribed to the molecular characterization compared to other issues concerning the safety assessment of GMOs [Hernandez, et al. 2003].

The EFSA GMO Panel accepts Southern Blot analysis to establish the stability of a transgenic insert [EFSA 2009]. Southern Analysis as well as PCR methods are commonly used to assess the genetic stability of a plant. [OECD 2010]. Southern blot analysis is the most commonly used method to characterize transgene loci. However, Southern blotting is not suitable for the detection of SNPs or very minor rearrangements: Only major changes can be detected by Southern blotting. However, minor changes have an important impact on the genetic stability of a plant [Kohli, et al. 2003].

Small changes in the construct can lead to unintended effects in the plant, concerning its properties, ingredients or its morphology [Moch 2006]. It has also been suggested that the genetic arrangement has an influence on the epigenetic regulation of the plant [Makarevitch, et al. 2007]. In addition, incorrect results of quantification may be caused by differences in genomic alleles [Broothaerts, et al. 2008].

In the case of genetic instabilities, the natural mutation rate must be considered since genome alteration may occur in all plants during the progeny process. Mitotic and meiotic cell division (as well as the prior processes), and the transmission of genes are all possible causes of a genotype alteration. Base pair incorporation during DNA replication or chromosome doubling can cause spontaneous mutations and crossing over (during meiosis) can also lead to genome modifications. Mitotic instabilities are rare after vegetative proliferation of GM crops. Genetic stability also depends of the transgene and the surrounding DNA [OECD 2010, Neumann, et al. 2009].

Transformational DNA modifications are not part of genetic stability. But in this context it is important to mention the transformational DNA modifications before explaining the post-transformational modifications which belong to the concept of genetic stability.

2.2.1. Transformational DNA modification

In recent years, it could be shown that during the production of transgenic plants undesired DNA changes (e.g. rearrangements in the target locus but also elsewhere in the genome) occur [Wilson, et al. 2004 & 2006]. The DNA in the plasmids used for the transformation into plants can be altered during the production of the final GMO product. Generally, the creation of transgenic crops should result in one copy of the desired transgene without further modifications to the DNA construct. However, different scenarios can occur during the transformation process resulting in, multiple copies of the insert, translocations or deletions, rearrangements of the transgene or the surrounding DNA, as well as,

unintentional insertion of “filler-DNA” from either vector or chromosomal DNA [Neumann, et al. 2011, OECD 2010, Windels, et al. 2001]. The type of modification also depends on the transformation method: *Agrobacterium*-mediated transformations can lead to tandem repeats or incomplete DNA integration, as well as, rearrangements or the insertion of plasmid backbone sequences. The latter can also happen with direct methods such as particle bombardment or electroporation causing the insertion of bacterial chromosomal DNA. Multiple copies of the transgene construct can be attributed as well to particle bombardment [OECD 2010]. Back-crossing is used to remove a high number of these undesired mutations [Wilson, et al. 2006].

The presence of these DNA changes during the integration process is relevant to the ERA as new DNA formations may lead to a number of complications: New substances in the GMO, altered levels of ribonucleic acid (RNA), and therefore altered proteins [OECD 2010]. It is difficult to control the transgene integration into the plant. Current methods only enable a random integration [Hernandez, et al. 2003].

Hernandez et al. (2003) detected a truncation at the 3´ end of the Cry1A(b) gene of MON810 produced through particle bombardment [Hernandez, et al. 2003]. Windels et al. (2001) found numerous rearrangements at the 3´- NOS junction as well as at the flanking region of soybean event 40-3-2 (Roundup Ready) [Windels, et al. 2001]. Maize event MON863 produced through particle bombardment is also affected and contains two different superfluous transgenic DNA sequences [Wilson, et al. 2006].

All described findings go back to the transformation process during the creation of the GMO. Newer experiments also indicate DNA rearrangements after the transformation process during the cultivation of GM plants.

2.2.2. Post-transformational DNA modification

The post-transformational stability of commercial transgenes and their flanking regions is not well-studied and the existing studies show different results. However, since DNA rearrangements after the transformation may influence the genetic stability of a plant further investigation is likely to be important [Neumann, et al. 2011].

Kohli et al. (1999) characterized transgenic rice lines. The experiments demonstrated an incomplete palindrome in the cauliflower mosaic virus 35S promoter in over 30% of all rearrangements suggesting a hot spot for rearrangements. The palindrome sequence has a hairpin secondary structure which may indicate undesired DNA recombination [Kohli, et al. 1999].

Choffnes et al. (2001) examined the genetic stability of the transgene soy line B1-10. It was the first study reporting genetic instabilities on the genomic level in a GM plant produced via particle bombardment. The rearrangements concerning the casein transgene were observed in the second as well as in the third plant generation. The progeny always displayed fewer copy numbers of the transgene than the parents. Southern blotting was used for the experiments. Although the plants contained multiple copies of the transgene no gene silencing occurred. Furthermore, all plants were fertile and no phenotypic differences occurred. Choffnes et al. (2001) suggested that either interchromosomal or intrachromosomal recombinations are responsible for the instabilities. Interchromsomal recombinations might split up the multiple transgene copies whereas intrastrand recombinations might occur when the transgene copies are very close [Choffnes, et al. 2001].

Rosati et al. (2008) reported protein differences in MON810 maize from different regions and demonstrated instabilities at the genomic level. The insert was modified at the 3´end and the whole NOS terminator as well as parts of the Cry1A(b) gene were deleted. Thus, the expression results only in a partial Cry1A toxin. Furthermore, the “stop” codon of the gene was no longer present,

leading to a read-through transcript containing a new sequence of Cry1A(b) and genomic maize DNA. Hence the artificial sequence causes new proteins showing no homology with any other known proteins [Rosati, et al. 2008].

Ogasawara et al. (2005) detected DNA changes in the coding region of the transgene and the adjacent *Cong* genes of progenies of soy event Roundup Ready 40-3-2. According to the neutral theory of molecular evolution: Mutations are equally distributed across the whole plant genome. This theory is in contradiction to the theory that the insert and the flanking regions are more susceptible to mutations. It must be considered that the first theory does not refer to GMOs [Kimura 1983, Ogasawara, et al. 2005].

The findings of Ogasawara et al. (2005) however, confirm the uniform distribution mentioned in the neutral theory of molecular evolution. A total of 34 mutations were found in the transgene and 37 mutations were identified in the *Cong* genes. The mutation rate of the transgene was nearly the same as that of the hosting gene. Both mutation rates were very high, in the transgene one mutation per 1144 base pairs (bp) and in the *Cong* genes one mutation per 1079bp. Hence there were no marked differences concerning the mutation rate on the genomic level. This was not the case on the proteomic level, where big differences occurred. Only four amino acid substitutions were detected in the transgene whereas 25 substitutions were identified in the *Cong* genes. In the case of the transgene, the alterations were mostly silent mutations because the substitutions were present in the third base of the codons [Ogasawara, et al. 2005].

La Paz et al. (2010) investigated the genetic stability of maize MON810 by using Southern Blotting and DNA mismatch endonuclease assays. By Southern Blotting no rearrangements were observed. Furthermore, when performing the DNA mismatch endonuclease assays, no SNPs were detected in the transgene, while in the 5´ flanking region six SNPs were identified. The SNPs were more than 500bp upstream from the insert and therefore, do not interfere with the de-

tection and quantification of GMOs. The mutation rate in the flanking regions was in the normal range for a maize repetitive sequence.

Additionally, La Paz et al (2010) analyzed the DNA cytosine methylation in different stages of plant development by bisulfite sequencing. Significant differences of asymmetrical DNA methylation were found in the 5´ flanking region between different commercial MON810 varieties. Hence, these results highlight the influence of the genetic background on DNA methylation levels: high DNA methylation can lead to gene silencing. However, concerning gene silencing symmetrical DNA methylation plays a bigger role than asymmetrical methylation [La Paz, et al. 2010].

There are however, studies concerning the genetic stability of GMOs which show no genomic instabilities of the transgene and the flanking regions [Neumann, et al. 2011] over generations [Ben Tahar, et al. 2010, EFSA 2009].

Papazova et al. (2006, 2008) investigated the stability of transgenic *Arabidopsis thaliana* plants under oxidative stress and tissue culture stress. Furthermore, the effect of gene stacking was studied. Neither abiotic stress or tissue culture stress nor gene stacking had an effect on the genetic stability. In both cases no nucleotide changes were detected [Papazova, et al. 2006 & 2008].

Neumann et al. (2011) examined 567 individual seeds of MON810 on the molecular level. Real-time PCR with Scorpion primers in combination with Sanger sequencing was used to analyze the 5 ´- and the 3 ´ regions of the insert. No polymorphisms were detected in the flanking regions [Neumann, et al. 2011].

Miklos et al. (2007) could not detect alterations in the transgene or the surrounding DNA by analyzing transgene soy plants containing the Cry1A gene from *Bacillus thuringiensis* (Bt) [Miklos, et al. 2007].

Most studies focusing on the genetic stability of commercial GM crops carried out by applicants are not on the molecular level. However, there are several other methods next to Southern blot analysis which are suitable for the analysis of genetic stability [Papazova, et al. 2008, Neumann, et al. 2011]. Ideally, the genetic stability would be investigated using techniques such as Southern Analysis as well as techniques covering analysis at the molecular level. Thus, a reliable safety assessment concerning the genetic stability could be carried out.
Additionally, in most of the studies only the transgene and the flanking regions were analyzed, although the whole genome can be affected by the introduction of the transgene [Padgette, et al. 1995]. For the official detection and quantification of GMOs it is state-of-the-art to use primers in the border regions. Therefore, the transgene and the surrounding DNA are an important target region as mutations in this region could impede event-specific real-time PCR [Neumann, et al. 2009].

Nevertheless it should be mentioned that genome rearrangements also occur in non-transgenic plants. Especially maize has a relatively high mutation rate because of the great number of mobile elements in its genome [Rosati, et al. 2008].

2.3. Zea Maize

The average mutation rate of maize genes is about $3x10^{-8}$ substitutions per side per generation. The rate of maize hypervariable microsatellites is estimated about $8x\ 10^{-4}$ substitutions per side per generation [La Paz, et al. 2010].
Zea maize belongs to the grass family (Poaceae). Maize plants are highly domesticated from the grass teosinte. This domestication has continued since ~10,000 years ago in Central America [Schnable, et al. 2009].

Maize is a wind-pollinated, monoecious species. It has separate staminate (tassels) and pistillate (silk) flowers. Self- and cross-pollination are generally possible. The frequencies are dependent on proximity and other influential factors on pollen transfer [Monsanto n.d.]. Without suitable human assistance maize plants

are not able to survive. Furthermore, maize plants are not winter hardy. Therefore, cross-pollination of maize is of reduced priority in Europe, except, in countries with very mild winters. Generally, outside cultivated or disturbed areas maize is not present in Europe [EFSA 2009]. Fig. 2 illustrates the development from teosinte´s few fruitcases to typical modern maize kernels.

Figure 2: Crossing for kernels [Gewin 2003], Photo from John Doebley

Zea maize is an annual crop. Tasselling, silking, and pollination are known to be critical in maize development. Furthermore, moisture and fertility stress have a major impact on the yield [Monsanto n.d.].

In the Maize Genome Sequencing Project from 2005 – 2008 the whole maize genome was sequenced [Anonymous 2011]. It has a size about 2.3 gigabases and contains 10 chromosomes (n=10, 2n=20). Therefore it is the largest plant genome ever sequenced. The size of the maize genome is due to lots of long terminal repeat retrotransposons. In the past the whole *Zea maize* genome was duplicated. Thus, returning to a diploid state was connected with several chromosomal breakages and fusions [Schnable, et al. 2009].

Maize is the second-most common GM crop after soybean. Today, the agricultural biotechnology industry does not only produce organisms containing single GM events: Conventional breeding is used to stack transgenic traits by crossing GM plants [Spök, et al. 2007].

2.4. Stacked events

In recent years an increasing number of stacked events have been announced under Regulation No. 1829/2003/EC and Directive 2001/18/EC [Spök, et al. 2007].

Stacked events are hybrids combined by conventional breeding. They contain two or more transgenic traits [EFSA 2007, Spök, et al. 2007]. Another possible way to stack events is by re-transforming a GM plant with additional transgenes [Wilson, et al. 2006]. But this possibility is not considered in the definition of stacked events by the EFSA [EFSA 2007]. However, in both cases transformation-induced mutations are likely to occur because of multiple insertions [Wilson, et al. 2006].

Stacked events have to undergo a standard authorisation procedure including the ERA [Spök, et al. 2007]. Combinations of transgenic traits increases the complexity of the ERA process because more undesired scenarios may occur. First, it should be verified that the independent single events present in the stacked event are safe. Furthermore, the combination of events must be stable, meaning that no interactions between the events should occur [EFSA 2011].

The EFSA authorised a special risk assessment for plants created by crossing to stack different GM events. Plants produced by crossing an EU-authorised event with non-GM events requires no extra risk evaluation. Furthermore, the EFSA determined that the risk assessment of a stacked event consisting of events approved in the EU should focus on three main points:

- the genetic stability of such plants
- the expression level of the transgene events
- unintended interactions between the stacked events [EFSA 2007].

Depending on the results of this studies further toxicological or nutritional experiments may be necessary [EFSA 2011].

The genetic stability of the transgenes is generally verified by Southern Blot analysis. Although the use of this method is under discussion because it cannot identify point mutations, its use is accepted and recommended by the EFSA. Newer EFSA guidance documents also recommend PCR analysis of the organism´s genetic stability, but this is not mandatory.

However, there is no detailed guidance on how to assess the genetic stability of inserted traits over several generations. Only expression analysis of the inserted transgenes is suggested to identify possible instabilities [Spök, et al. 2007]. Furthermore, it is not clear if any changes in the genome occur and if they are relevant for the assessment of stacked events. The EFSA concentrates only on DNA changes in the transgenes and the flanking regions [Latham, et al. 2006].

Additionally, stacked events cause new GMO quantification problems. The identified percentage content of individual GM events cannot be clearly assigned because the events can belong to single events as well as to a stacked event. The following example illustrates the problem: A mixed food sample is analyzed. The detected percentage GMO content is 1.6% which belongs to two GM events. If 0.8% belong to NK603 and 0.8% belong to MON810, the threshold of 0.9% (max. GMO content in food in the EU) would be exceeded with a total of 1.6%. But if the two events belong to one stacked event (NK603 x MON810), the GMO content would be 0.8% and therefore be accepted.

The mentioned hypothetical problematic scenario is the reason for the rejection of stacked events by Austria. Austria states that as long as no specific detection method to distinguish between a stacked event and its single events is provided, stacked events should not enter the market [National Authority, Austria 2013].

3. Material and Methods

3.1. Materials

3.1.1. Object of investigation

In this Master project, the stacked event 4421VT3 maize was investigated to verify the genetic stability of the GMO. 4421VT3 consists of three transgenic events, MON810, also known as YieldGard® Corn Borer maize (Cry1Ab), Roundup Ready® maize NK603 (CP4 EPSPS) and YieldGard Rootworm® maize MON863 (Cry3Bb1) [Monsanto 2002, 2003 & 2004]. Seeds were obtained from the USA.

The stacked event results from conventional breeding whereas the parental lines were produced by genetic modification (particle acceleration method) [Monsanto n.d.].

Furthermore, the stacked event 6831RHXT served as a control for NK603 experiments since it also contains the event NK603.

3.1.2. Kits

The PCR reagents and PCR primers used were of analytical grade. Unless stated otherwise, all kits were used according to the manufacturer`s recommendations:

- TA cloning® kit (Invitrogen)
- Easy Prep Pro miniprep kit (Biozym)
- TOP10 competent cells (Promega)
- Wizard DNA cleanup (Promega)
- illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare)
- E.Z.N.A.® Gel Extraction Kit (OMEGA)
- Type-it HRM PCR Kit (Qiagen)
- GoTaq® DNA Polymerase (Promega)
- GoTaq® Hot Start Polymerase (Promega)

3.1.3. Primer

Primer	Type	Sequence
3´MON810fwd	Normal forward	5´-CCAAGCACGAGACCGTCAA-3´
3´MON810rev	Normal reverse	5´-GCTCGCAAGCAAATTCGGAA-3´
3´MON810sc	Scorpion reverse	5´-[F]CGCGGCGAGGACTTTCGGTAGCCGCG[Q][HEG]GCTCGCAAGCAAATTCGGAA-3´
VW01	Normal forward	5´-TCGAAGGACGAAGGACTCTAACG-3´
VW03	Normal reverse	5´-TCCATCTTTGGGACCACTGTCG-3´

[F] Fluorophore, [Q] quencher, [HEG] stopper, the F/Q of 3´MON810sc are Fluorescein/BHQ™

Table 1: List of primer names, type and sequence for the investigation of the event MON810

Primer	Type	Sequence
NK603 NOS-Gen_fwd	Normal forward	5´-ATGAATGACCTCGAGTAAGCTTGTTAA-3´
NK603 NOS-Gen_rev	Normal reverse	5´-AAGAGATAACAGGATCCACTCAAACACT-3´
NK603 Act_fwd1	Normal forward	5´-TATGCTTGAGAAGAGAGTCGGG-3´
NK603 Act_rev1	Normal reverse	5´-TTGGGCCACCTTTTATTACCG-3´
NK603_CP4/e35 fwd	Normal forward	5´-GTTGCCGGTCTTGCGATGAT-3´

Table 2: List of primer names, type and sequence for the investigation of the event NK603

Primer	Type	Sequence
M13-fwd	Normal forward	5´-AAGCCGAATTCTGCAGATATC CATCACACTGGCGGCCGCTCGA GCATGCATCTAGAGGGCCCAATT CGCCCTATAGTGAGTCGTATTAC AATTCACTGGCCGTCGTTTTACA A-3´
M13-rev	Normal reverse	5´-CAGGAAACAGCTATGACCATG ATTACGCCAAGCTTGGTACCGAG CTCGGATCCACTAGTAACGGCC GCCAGTGTGCTGGAATTCGGCT T-3´

Table 3: List of primer names, type and sequence for verifying the M13-sequences

3.1.4. Equipment list

- Mettler AT261 Delta Range (Analytical balance)
- Sartorius Bp2100 (Balance)
- Memmert (Heating cabinet)
- Centrifuge 5415 D (Eppendorf)
- Gilson Pipettes pipetman (1000µl, 200µl, 10µl)
- MS2 Minishaker (IKA)
- Thermomixer comfort (Eppendorf)
- Thermomixer 5436 (Eppendorf)
- EBA12 Centrifuge (Hettich)
- Varioklav Autoklave H+P (Varioklav)
- Vakuum pump Neuberger (Freiburg) + 20 x Distributor (Promega)
- Nanophotometer™ (IMPLEN)
- Labofuge 400 (Heraeus instruments)
- Electrophoresis Power Supply- EPS 601 (Amersham Pharmacia Biotech)
- MR-5400 Mikrowave (Hitachi)
- HE 99X max. Submarine unit (Hoefer)

- Refrigerator (Liebherr, +4°C)
- Freezer apparatus (Constructa Serva, -20°C)
- Freezer apparatus, UltimaII (Revco, -80°C)
- Mastercycler epgradient S (Eppendorf)
- Rotor-Gene RG-3000 (Corbett Research)
- Inkubator, CERTOMAT BS-1 (Bram Biotech International)
- Nanophotometer™ from IMPLEN

3.2. Methods

3.2.1. Basics

3.2.1.1. DNA-extraction and purification

For the planned investigations 132 single grain DNA samples were produced following the DNA extraction protocol.

Individual seeds were homogenized using a garlic crusher. Afterwards, 150mg crushed seeds were homogenated with 820µl TNE-buffer [10mM Tris (pH 8), 150mM NaCl, 2mM EDTA (pH 8), 1% SDS], 150µl 5M Guanidine-HCl and 30µl proteinase K and incubated overnight at 60°C under constant shaking. Subsequently the homogenates were centrifuged at 13200rpm. 560µl of the supernatant were transferred into another tube and 300µl chloroform (<99%) were added. The mixture was then vortexed for 20s and subsequently centrifuged at 13200rpm for 8min to separate the phases. 500µl from the upper aqueous phase were transferred into a new tube. 2µl RNAse (8µg/ml) were added and the samples were incubated under constant shaking at 60°C for 30min.

The genomic DNA extracts were then purified with the Wizard DNA cleanup kit from Promega. The pre-elution was performed with 20µl ddH_2O. The elution was then carried out with 70°C 10mM Tris (pH 7.4) for 10min.

3.2.1.2. Photometer

After purification the DNA quantity as well as the DNA purity were measured photometrically using the Nanophotometer™ from IMPLEN. The measured DNA

concentrations ranged from 42ng/µl to 140ng/µl with an average concentration of 79.3ng/µl (only suitable DNA extracts included).

Additionally, DNA purity was measured by using the protein contamination ratio 260nm/280nm as well as the 260nm/230nm ratio of other contaminants. Optimum purity values were 1.8 for the protein contamination ratio and 2.0 – 2.2 for the ratio of other contaminants.

The outcomes of the planned analysis by real-time PCR and subsequent HRM analysis would depend strongly on the DNA quality used for the experiments [Madi, et al. 2013]. Therefore, an accurate review of the DNA purity was necessary.

3.2.1.3. *Gel electrophoresis*

To control the DNA purity and the length of bands from the DNA extracts, the genomic DNA samples were loaded on 1% agarose gel and run in electrophoresis apparatus for 22min at 140 volts. Fig. 3 shows 18 4421VT3 DNA samples on a 1% agarose gel photographed under UV light.

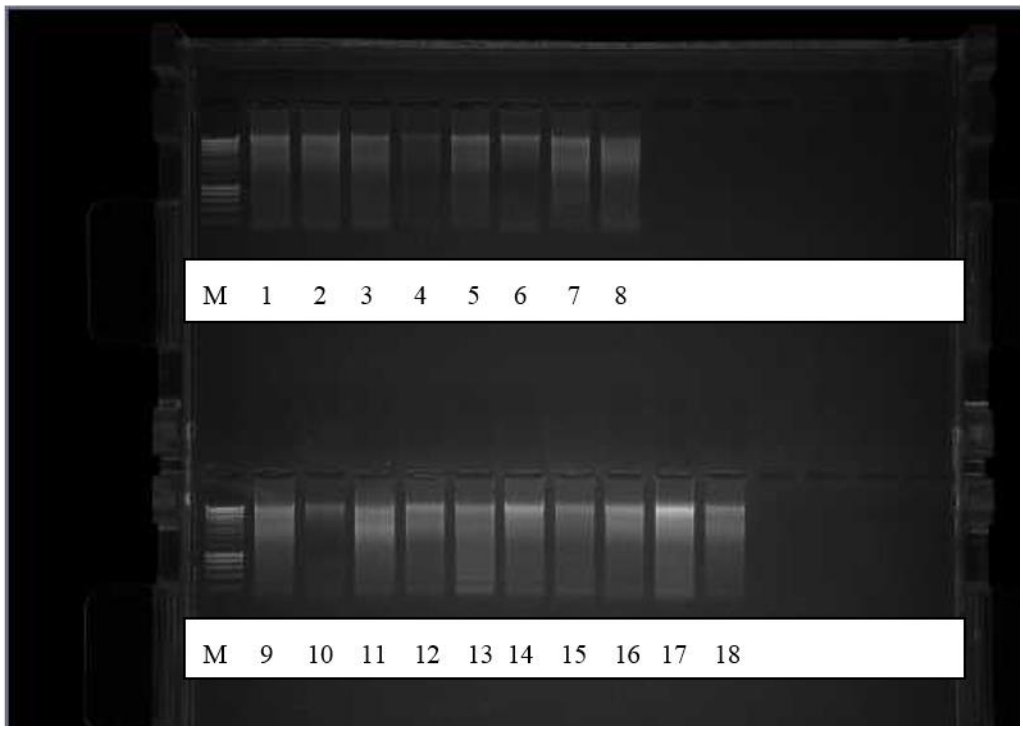

Fig. 3: 1% agarose gel showing the DNA purity

Fig. 3 shows, on the left side, the DNA markers marked with M and then 18 single grain maize DNA samples numbered from 1 to 18. The marker ranges from 200bp at the bottom to 10kb at the top. DNA purities and concentrations differ slightly from each other, e.g. sample no. 11 has a lower DNA purity in comparison with sample no. 10 while the DNA concentrations are about the same. An illustration of DNA concentration differences can be seen in samples no. 4 and 5: The band from sample no. 5 is broader and clearer in comparison with the band of sample no. 4. Some sample slots can be seen to contain little DNA due to the DNA molecules becoming stuck in rough areas of the gel. Furthermore, there are small impurities in the area under 200bp.
However, altogether fig. 3 shows clearly that the DNA is clean and has only weak degradation.

After verifying the DNA purities, all samples were immediately stored at - 20°C until use. The DNA preps described above were used for both the investigation of the event MON810, and, for the analysis of NK603.

Gel electrophoresis was further used to check PCR products. For the verification of the band length, 2.5% agarose gels were produced. The running conditions were the same as above.

3.2.1.4. *PCR*

Qualitative PCR was used to check if the maize variety contained the transgenes and to verify that the transgene was also detectable in samples with lower DNA content. 20 to 40% of the samples underwent an event-specific test by qualitative PCR. For verification the events MON810 and NK603 were selected. The primers VW01 and VW03 (for event MON810) and NK603 NOS-Gen_fwd and rev (for event NK603) were used for the PCR analysis with subsequent agarose gel (2.5%) evaluation. All tested samples contained the MON810 transgene but in none of the samples was the event NK603 detected using the primers NOS-Gen_fwd and rev.

Furthermore, PCR was used for cloning and for mapping of the NK603 deletion because the event NK603 could not be demonstrated to be present by detection with the verification primers.

Generally, reaction approaches had a volume of 20µl and were composed as follows if not stated otherwise (table 4):

Component	**Final Volume**	**Final Conc.**
5X Colorless GoTaq® Reaction Buffer	4µl	1X(1.5mM $MgCl_2$)
dNTP (40mM)	0.5µl	1mM
Each Primer (100µM)	0.5µl	2,5µM
GoTaq® DNA polymerase (5u/µl)	0.3µl	1.5u
Template DNA	1µl	<0.2µg/20µl
ddH_2O to	20µl	-

Table 4: Reaction approach for PCR analysis

PCR programme:

a) Initialization step: 94°C – 10min
b) Denaturation step: 94°C – 30sec
c) Annealing step: 60°C – 30sec
d) Elongation step: 72°C – 30sec
e) Final elongation: 72°C – 1min
f) Final hold: 16°C – hold

The annealing steps and cycle numbers varied for different primers. Primers used for the experiments are listed in tables 1, 2 and 3. The annealing temperature varied from 58°C – 62°C and the repetition of steps b) to d) varied between 35 – 40 cycles.

3.2.1.5. *Cloning*

The first step in cloning was a PCR run with a subsequent agarose gel (2.5%) check of the PCR products. Cloning was performed with the TA cloning® kit (Invitrogen). After ligation into the pCR 2.1 vector the ligation product was stored overnight at 4°C.

Prior to the transformation step agar plates were produced by adding 40g LB-Agar (Roth) into one litre distillate water. The mixture was autoclaved for 20min. After cooling down to ~50°C 2ml ampicillin [10mg/ml; (Sigma)] were added. This mixture was used to pour agar plates in petri dishes. After curing the plates were stored in the refrigerator at 4°C.

3µl of the ligation product were transformed into 50µl JM109 Competent Cells (Promega). Afterwards 450µl SOC-medium were added to the transformation product and the bacterial culture was incubated at 37°C, 225rpm for 1h.

Blue-White selection was used to identify positive colonies. Therefore the agar plates were covered with 80µl XGAL [20mg/ml; (Sigma); in Dimethylformamide (Merck)] and 16µl IPTG [0.1M; (Sigma)] before spreading out the culture samples. The streaked agar plates were stored overnight at 37°C. Positive colonies were white whereas blue indicated negative colonies.

Positive colonies were removed and subsequently grown in 5ml LB-medium overnight. Plasmid DNA of positive colonies was tested for the right length of the insert by PCR and subsequently electrophoresis using the specific primers and M13-primers. M13-primers were used to verify that the M13 sequence necessary for sequencing is present in the plasmids.

For this PCR analysis a Hot Start system (table 5) was used:

Component	**Final Volume**	**Final Conc.**
5X Colorless GoTaq® Flexi Buffer	4µl	1X(1.5mM $MgCl_2$)
$MgCl_2$ solution, 25mM	1.6µl	2mM
dNTP (40mM)	0.5µl	1mM
Each Primer (100µM)	0.5µl	2.5µM
GoTaq® Hot Start polymerase (5u/µl)	0.3µl	1.5u
Template DNA	1µl	<0.2µg/20µl
ddH_2O to	20µl	-

Table 5: Reaction approach for PCR analysis after cloning

The temperature programme as well as the cycle number was the same as in the PCR for the ligation (according to the temperature programme under sub-item PCR).

After verifying the colonies, those verified to contain the right length of the insert as well as the right length of the M13-sequence were isolated with the Easy Prep Pro miniprep kit.

3.2.1.6. *Gel extraction*

For gel extraction the required bands were cut out of the gel under UV-light using a scalpel. Then the gel bands were transferred into Eppendorf tubes. After weighing the bands, the E.Z.N.A.® Gel Extraction Kit (OMEGA) was used according to the recommendations to extract and purify the DNA. Subsequently the DNA concentration was measured using a Nanophotometer™ from IMPLEN.

3.2.2 Screening methods

3.2.2.1. Real-time PCR using Scorpion primers

Real-time PCR was conducted on a Rotor-Gene RG-3000 (Corbett Research). 2 different approaches were used and compared. The Type-it HRM PCR Kit (Qiagen) was used in 16µl reaction volume with following reaction conditions:

Component	**Final Volume**	**Final Conc.**
HRM PCR Mastermix (2X)	8µl	1X
3´MON810fwd (10µM)	1µl	0.625µM
3´MON810sc (10µM)	0.2µl	0.125µM
Plasmid DNA (10^{-4}ng/µl)/ Genomic DNA	1.6µl	10^{-5}ng/µl/ <0.16µg/16µl
ddH_2O to	16µl	-

Table 6: Reaction approach for real-time PCR with Type-it HRM PCR kit

Furthermore a self-made mastermix was produced as follows:

Component	**Final Volume**	**Final Conc.**
5X Colorless GoTaq® Flexi Buffer	4µl	1X(1.5mM $MgCl_2$)
SYTO (33.3mM)	2.4µl	4mM
$MgCl_2$ solution, 25mM	2.8µl	3.5mM
dNTP (40mM)	0.1µl	0.2mM
Each Primer (10µM)	0.2µl	0.1µM
GoTaq® Hot Start polymerase (5u/µl)	0.3µl	1.5u
Template DNA	2µl	<0.2µg/20µl
ddH_2O to	20µl	-

Table 7: Reaction approach for real-time PCR with self-made mastermix

The temperature profile as well as the cycling profile were the same for both approaches:

a) Initialization step: 95°C – 5min

b) Denaturation step: 95°C – 10sec

c) Annealing step: 55°C – 30sec

d) Elongation step: 72°C – 20sec

e) HRM 70°C – 95°C

Steps b) to d) were repeated 55 times.

The Type-it HRM PCR Kit contains the fluorescent dye EvaGreen whereas the self-made mastermix contained the fluorescent dye SYTO. The spectral properties of these fluorescent dyes are very similar to those of SYBR Green.

Unimolecular, singular Scorpion primers (3´MON810fwd and 3´MON810sc) consisting of a forward and a reverse PCR primer sequence as well as a PCR stopper, a specific probe sequence and a fluorescence detection system with fluorophore (Fluorescein) and quencher (BHQ^{TM}) were used for the analysis of the maize event MON810 by real-time PCR.

The PCR stopper prevents PCR read-through of the probe sequence. During the PCR, an amplicon is generated which contains a sequence complementary to the Scorpion probe. This specific probe sequence binds to the complementary sequence within the amplicon. Thus, the Scorpion loop is opened resulting in the formation of a signal [Madi, et al. 2013, Neumann, et al. 2011]. Fig. 4 shows the Scorpion probing mechanism.

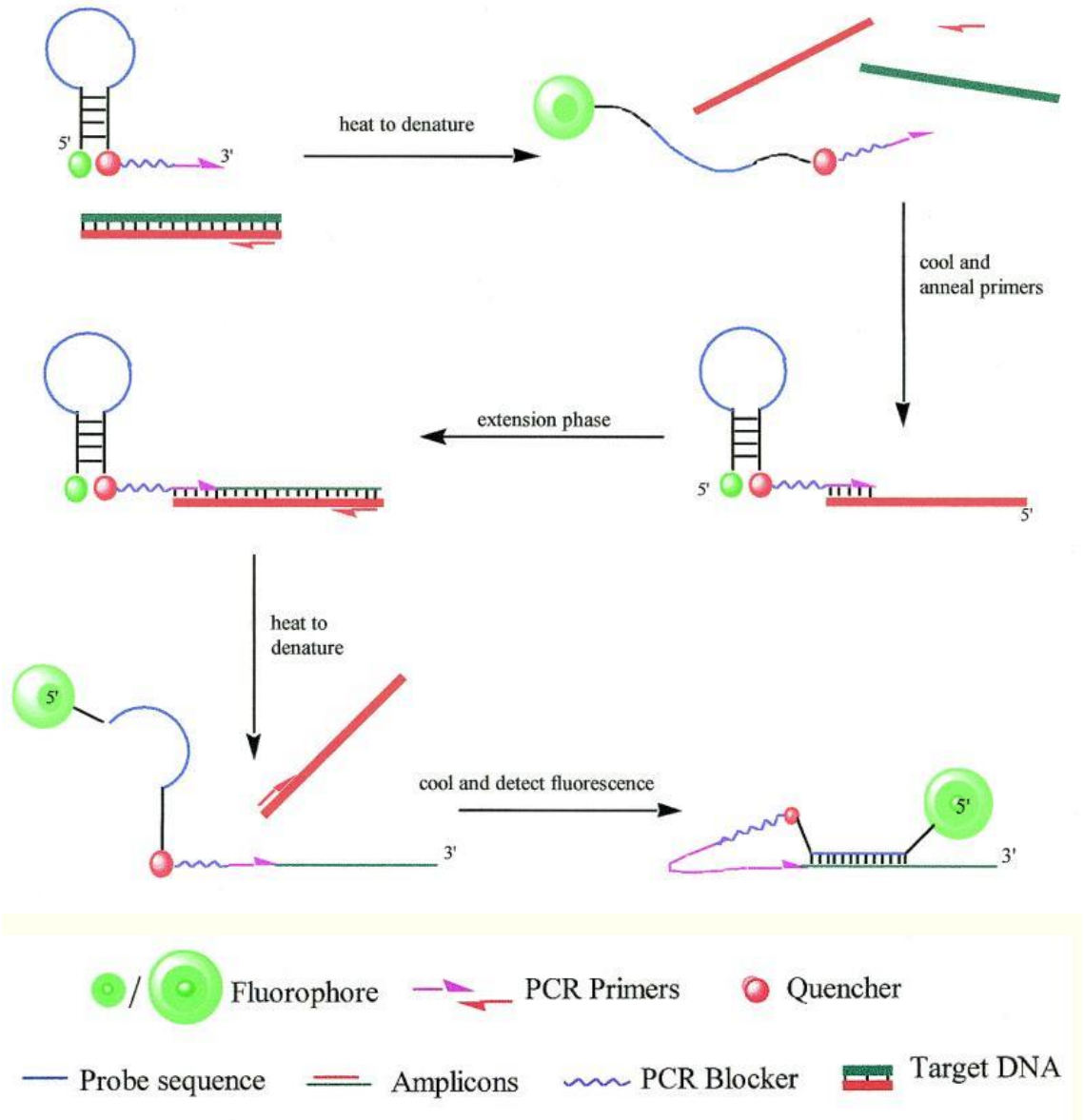

Fig. 4: Scorpion probing mechanism [Thellwell, et al. 2000]

The first step shown in in fig. 4 illustrates the initial denaturing of the target sequence and the Scorpion stem sequence. The annealing of the Scorpion primer to the target DNA then follows in step 2. In the extension phase (step 3) double-stranded DNA is produced which becomes denatured in step 4. Thereby a single-stranded molecule, connected to the Scorpion primer is created. The single-stranded molecule is the target sequence for the Scorpion probe which binds to it in an intramolecular manner during step 5 [Thellwell, et al. 2000].

In every real-time PCR run two different fluorescence signals were measured (in two different channels). The Scorpion signal was measured in the red channel (excitation: 625+/- 5nm; detection: 660+/-10nm) whereas the fluorescence of EvaGreen or SYTO was measured in the green channel (excitation: 470+/-10nm; detection 510+/-5nm.

3.2.2.2. HRM

HRM is a post PCR method used for high throughput mutation scanning among other uses. It is based on DNA melting. A high-copy number of the target sequence and a double-stranded DNA binding dye is necessary for this technique. Melted PCR products have individual melting and disassociation behaviours which are observed by measuring the fluorescence decrease during melting. Thus, differences between homozygous as well as heterozygous samples could be distinguished. In the first case the melting temperature differs whereas in the latter the shape of the melting curve changes. Melting behaviour depends of the length and the GC content of the target sequences [Lehmensiek, et al. 2008].

HRM was performed in the same real-time PCR run as the Scorpion analysis. Thus for the HRM the Scorpion primers were used too. The rate temperature increase during melting was 1°C per second. The initial temperature was 70°C and the final temperature was 95°C. By using the LC480 software (version 1.3; Roche) the melting curves were normalized and temp-shifted to make the curves comparable. After selecting a reference, the software gave HRM scores (Confidence values) to evaluate the results [Madi, et al. 2013].

3.2.3 Sequencing

Sequencing was performed by the Institute for Medical Microbiology, Core Unit Molecular Biology by using the BigDye Fast cycle sequencing protocol described by Platt, et al. (Platt, et al. 2007).

Sequencing results were obtained by Sanger sequencing using M13 primers. Furthermore, the M13 sequences necessary for sequencing were present in

produced plasmids as the cloning vector already contained these sequences. To avoid sequencing errors the method included both forward and reverse sequencing. This ensured a high reliability of the sequencing results. In cases of nucleotide changes confirmed by forward as well as reverse sequencing the sequencing chromatograms were checked in detail.

3.3. Description of the investigation

Different systems were used to study selected sequences in the commercial GM maize. DNA sections commonly used for the official detection and quantification of GMO were chosen for the experiments because DNA changes in these regions may cause false results in GMO control. Usually the transitions from the original maize genome into the transgenic construct at the 3´ end and 5´ end are used for specific analysis in GMO detection. Further, Papazova et al. (2008) mentioned that the T-DNA flanking regions are possibly more susceptible to mutations through genotoxic stress than other regions in the genome [Papazova, et al. 2008]. However, it may be that other areas are more affected by genetic instabilities.

Prior to the analysis the detectability of the transgenes was checked. For the demonstration of the event MON810 the primers VW01 and VW03 were used. Further to this, for the verification of the event NK603 the primers NK603 NOS-Gen_fwd and NK603 NOS-Gen_rev were used.

The event MON810 was studied at the T-DNA flanking region using Scorpion primers as well as HRM analysis. NK603 was examined with conventional PCR. The event MON863 was not analyzed.

The following chapters explain the procedure of analyzing the genetic stability and present the obtained results.

3.3.1. Investigation of the event MON810

In this project, the presence or absence of small DNA alterations in the target sequence was investigated by a three-step procedure starting with the screening of a high number of individual maize seeds. Thus, the first screening was performed with genomic DNA by using real-time PCR with Scorpion primers as well as HRM analysis. Second, deviating genomic samples were cloned into plasmids and subsequently analysed using the same methods. The third step was to verify possible mutations by Sanger sequencing. Only samples with scores in the first and the second screening pointing to mutations entered step 3. Fig. 5 shows a summary of the schema.

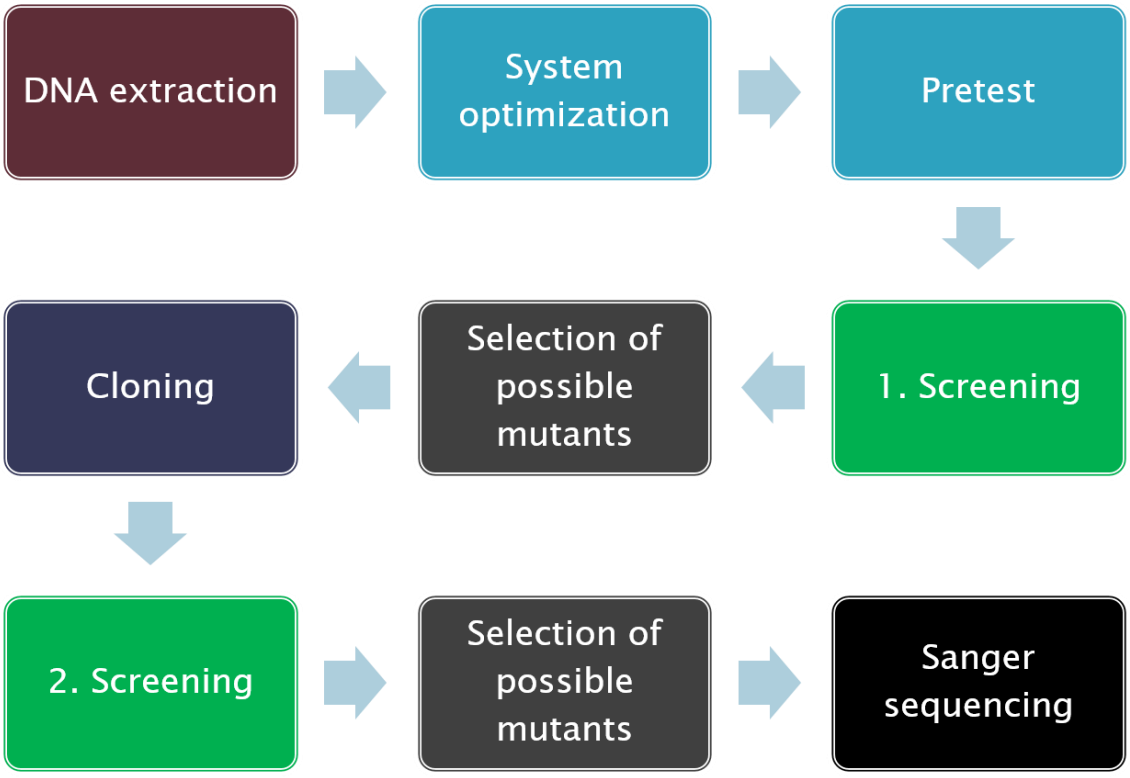

Fig. 5: Schema of the analysis of the 3´ MON810 target region

Fig. 5 illustrates the basic procedure for the analysis of the event MON810. The individual steps are described in detail below, except system optimization. Sys-

tem optimization means optimization of the PCR reagents and of the program, respectively. Sanger sequencing is the last step of the schema.

As mentioned above real-time PCR with Scorpion primers as well as HRM analysis were used to investigate the genetic stability of the MON810 transgene. These methods - especially when analysed with combined scores - can already detect small changes in DNA sequences. Southern Blots commonly used for the investigation of the genetic stability are not suitable to detect SNPs. Furthermore, a large number of DNA samples can be tested if the two systems are performed in a single PCR run. Mutations in the analysed sequence, particularly in the high sensitive Scorpion probe sequence result in differing real-time PCR plots [Neumann, et al. 2011].

In case of the Scorpion sequence the evaluation was carried out by mathematical analysis based on Ct values. It was assumed that mutations in the probe sequence as well as mutations in the primer regions would result in higher Ct values.

HRM melting curves usually differ from wild type samples because of their modified melting behavior. Therefore samples with a different melting pattern can be recognized by very low Confidence values. Below, the evaluation is described in detail.

Combining these systems results in a highly sensitive method for the detection of the SNPs that may impede GMO detection [Madi, et al. 2013]. Weaknesses of HRM analysis can be compensated with the Scorpion system and vice versa. Ideally, both systems provide the same assessment of a sample. But the combination of the two systems at the same time allowed a precise comparison of real-time PCR with Scorpion primers and HRM analysis regarding their ability to identify SNPs.

To avoid interactions, two dyes which fluoresce at different wavelengths were selected for the real-time PCR with Scorpion primers and the HRM analysis. The reverse Scorpion primer was equipped with Fluorescein as reporter dye, whereas at the same time SYTO or EvaGreen was taken for the HRM analysis.
In addition, a third system for checking the DNA integrity was used. Therefore, the fluorescence increase of either SYTO or EvaGreen was measured in a different channel during real-time PCR. Normal amplification ensures intact DNA while high Ct values indicate impurities [Neumann, et al. 2011; Madi, et al. 2013]. However, this does not mean that samples with different EvaGreen or SYTO amplification have inevitably denatured DNA. Also, larger mutations of a sequence can result in different curves.

The following procedure was used to detect SNPs. Samples with normal EvaGreen or SYTO amplification plots and curve deviations in both, Scorpion and HRM analysis, entered step 2 (second screening) and were subsequently tested by Sanger sequencing (step 3) after passing step 2. Samples with a strong difference in only one system, either Scorpion or HRM, also entered step 3.

Real-time PCR and Scorpion analysis are quantitative methods. Thus they have a natural scattering influenced by artifacts and impurities of the DNA. In contrast, Sanger sequencing is only slightly or unaffected by artifacts. To exclude influences of artifacts or PCR errors, cloning was used. It was assumed that by producing a minimum of three plasmids of potential mutated samples, artifacts and PCR errors would be detected. In the end, sequencing was the final confirmation of SNPs.

As already mentioned above the analyzed target sequence lies directly at the transition from the introduced MON810 transgene to the maize genome and is therefore event-specific. For both, real-time PCR with Scorpion primers and HRM analysis the same target sequence was used. Fig. 6 shows the examined

target sequence of the Scorpion primer for the 3´junction of the MON810 transgene.

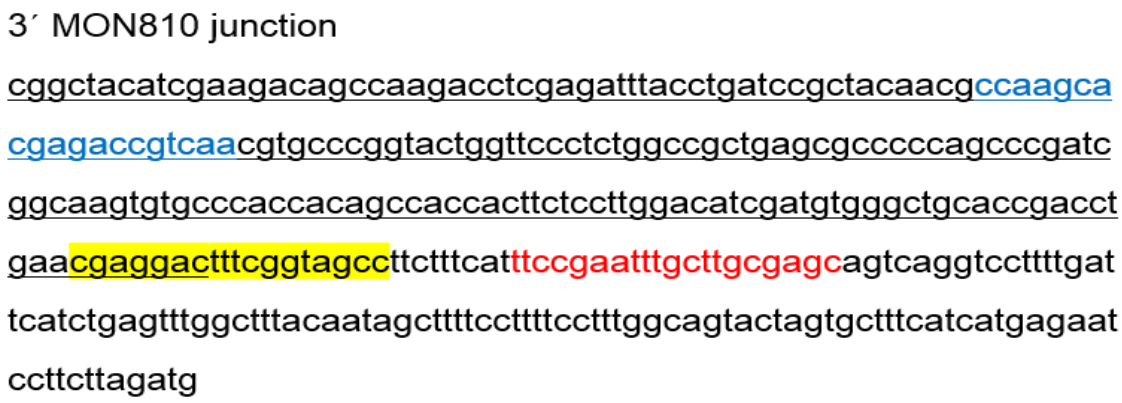

Fig. 6: Illustration of the target sequence of the Scorpion primer for the 3´ junction of the MON810 transgene [modified according to Neumann, et al. 2011]

In fig. 6 the forward primer is displayed in blue, the reverse primer in red and the related Scorpion probe is depicted in yellow. Furthermore, the sequence of the MON810 transgene construct at the 3´ end of the Cry1A(b) gene is shown in underlined letters and the Scorpion probe is located exactly at the T-DNA flanking region [Neumann, et al. 2011]. Neumann et al. demonstrated with PCR experiments and BLAST search that the present primer design is very specific and therefore enables highly accurate SNP detection [Neumann, et al. 2009].

The analyzed sequence contains 180 nucleotides. In order to determine the sensitivity of the methods, experiments were made with plasmids which had small changes in the target area. The latter was necessary because there are no known naturally occurring SNPs. Plasmid DNA has the benefit that no quality variations of genomic DNA extracts can influence the validation (Neumann, et al. 2011). However, it should be noted that cloned DNA possesses properties other than those of genomic DNA. Thus, results obtained from plasmid DNA cannot be directly compared with the results which were obtained with genomic DNA.

3.3.2. Investigation of the event NK603

Originally, the analysis of the event NK603 was supposed to run in the same manner as the MON810 investigation. As a first step a suitable location at the flanking regions had to be found to realise the plan. Before starting the analysis it was important to ensure that the 4421VT3 maize actually contained the event NK603.

Consequently, an event-specific PCR using the recommended primer pair listed in the protocol for NK603 quantification from the Joint Research Centre (JRC) Biotechnology & GMOs unit from the European Commission was performed. The specific primer pair NK603 NOS-Gen_fwd and NK603 NOS-Gen_rev (listed under the section Material) was designed to amplify a 108-bp fragment at the 3´ T-flanking region [JRC 2005].

However, the result of the check was that the 108-bp fragment could not be amplified by qualitative PCR (see results). Hence the original plan was not feasible. It was assumed that the event NK603 has a deletion at the examined locus. Therefore, the revised plan was to map the deletion by performing PCR using different primers.

4. Results

The result of this master project was that the events MON810 and NK603 show genetic instabilities in the stacked event 4421VT3 maize. Thus, the results indicate that post-transformational alterations occurred, although the single events present in 4421VT3 maize are assessed as safe by the EFSA [EFSA 2003, 2010 & 2012].

4.1. Results MON810

The sequencing results showed many different alterations in the 3´ MON810 target region. Before conducting the experiments it was assumed that if mutations occur, these would occur at the same locus in different samples. It was surprising to find many different SNPs in the T-flanking region. In some samples

that were cloned into plasmids as many as three different nucleotides at the same locus were found.

4.1.1. Scorpion PCR and HRM analysis in plasmids

For the sensitivity check three plasmids were used, one had a 1-bp deletion at the 3´ MON810 junction, the second one had a 2-bp deletion at the 3´ MON810 junction and the third one was a wild type plasmid according to the target sequence illustrated in fig. 6 [Neumann, et al. 2011]. Prior to the experiments the plasmids were diluted to 10^{-4}ng/µl because preliminary tests showed that this concentration is well suited to the proposed method.

A real-time PCR with Scorpion primers and subsequent HRM analysis (with conditions described in the Materials and Methods section real-time PCR with Scorpion primers and subsection HRM analysis) was conducted with the three plasmids mentioned above and in case of real-time PCR with Scorpion primers additionally with a genomic 4421VT3-Pool. The aim was to demonstrate that both systems are able to detect even minor changes in the target sequence. The 4421VT3-Pool consists of 20 different genomic DNA samples of 4421VT3 maize. Figures 7 and 8 show the results obtained with plasmids by real-time PCR with Scorpion primers (fig. 7) and HRM analysis (fig. 8).

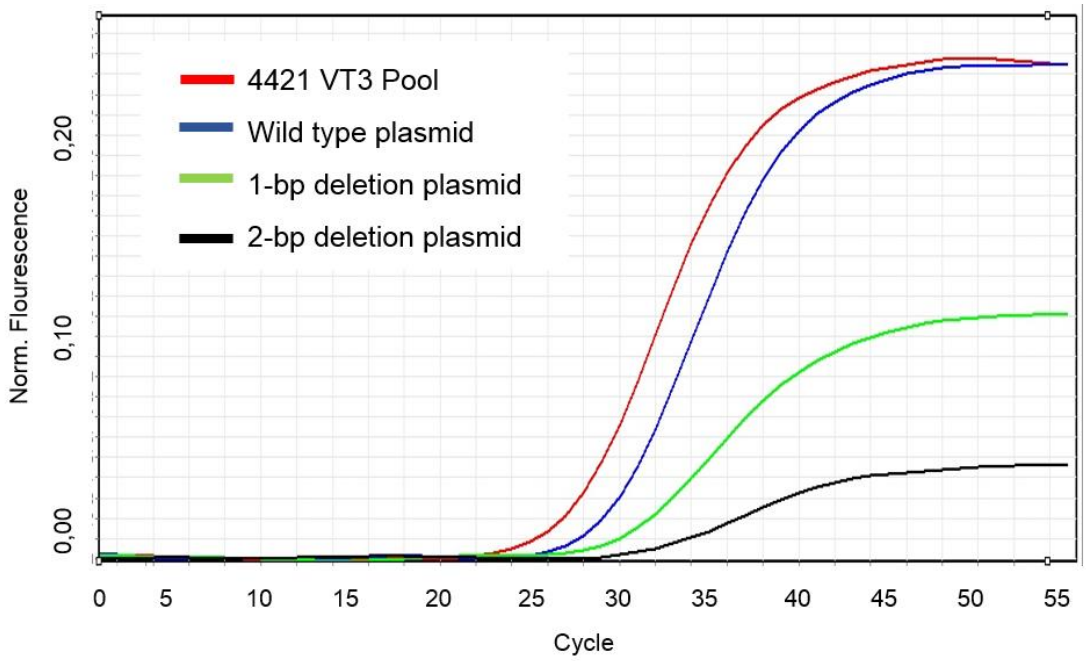

Fig. 7: Scorpion analysis with plasmids containing the MON810 target sequence

Fig. 7 shows clearly that a distinction between mutant and wild type is possible with Scorpion primers. Even the 1-bp deletion plasmid differs markedly from the wild type plasmid. The 2-bp deletion differs more from the wild type than the 1-bp deletion. The amplification curve of the 4421VT3-Pool demonstrates that genomic DNA and plasmid DNA result in similar amplification curves. This shows that the comparison of experiments with cloned DNA to those with experiments with genomic DNA is basically possible.

Neumann, et al. (2011) demonstrated by analyzes of mixtures from mutated and wild type plasmid DNA that the Scorpion method is sensitive enough to detect even heterozygous DNA alterations [Neumann, et al. 2011]. This is very important because heterozygous mutations can occur and remain undetected by other methods such as Southern blots or sequencing alone.

The next step was to clarify the sensitivity of the HRM analysis and to compare the Scorpion system with the HRM analysis. Fig. 8 shows the results of the sensitivity check obtained with HRM analysis.

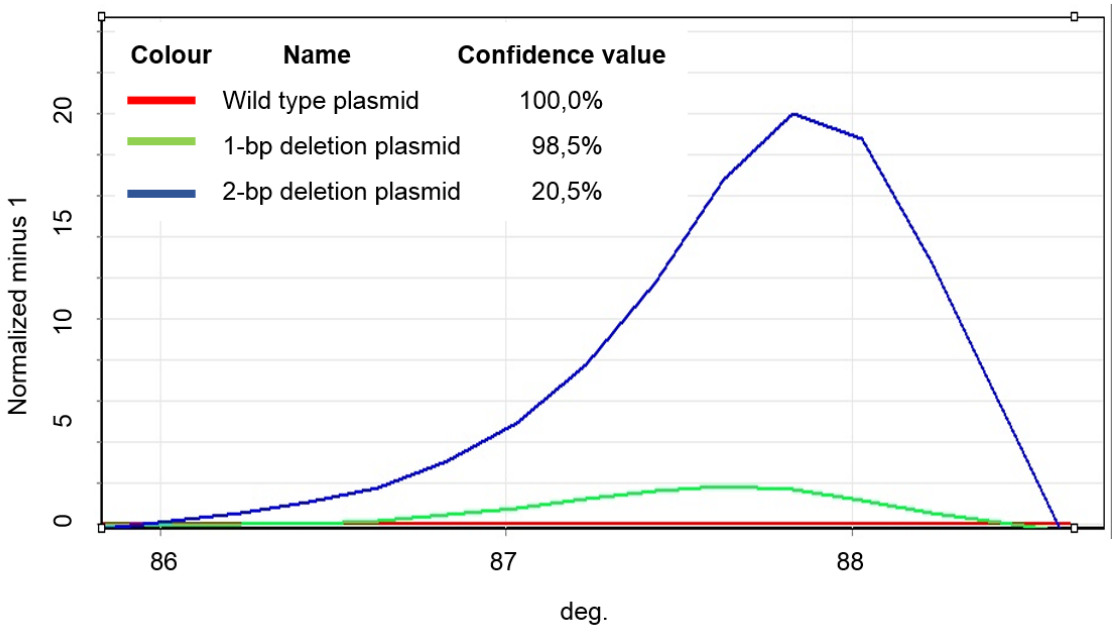

Fig. 8: HRM analysis with plasmids containing the MON810 target sequence

The difference graph illustrated in fig. 8 shows that HRM analysis is also suitable for the detection of SNPs. However, the distinction by means of confidence values is not as clear as expected. The melting pattern of the wild type plasmid served as reference for the other melting curves. The 2-bp deletion plasmid and the wild type plasmid differ markedly from each other, while the 1-bp deletion plasmid and the wild type plasmid differ only slightly from each other.

The visual evaluation of the difference graph shown in fig. 8 is also reflected by the confidence values. But a Confidence value of 98% indicates no real difference in HRM evaluation because such a difference is within the range of natural fluctuation of a genotype. To avoid false positive results, only Confidence values with a minimum difference of 50% were considered in the following analyses. However, the experiments show that even with small observed differences the possibility of SNPs may not be excluded.

Nevertheless, for confidence values the evaluation concept will remain the same. As before, it is assumed that the samples with the lowest confidence val-

ues are potential mutations, which must be verified by sequencing. The rationale behind this procedure is supported by the fact that greater alterations in the target sequence (2-bp deletion plasmid) result in lower confidence values than minor DNA changes (1-bp deletion plasmid).

4.1.2. First screening

The first screening served to filter out genomic samples which contained SNPs in the T-DNA flanking regions. For the first screening 2 x 50 4421VT3 samples with purified DNA were selected. Further, for each screening a real-time PCR was carried out with the prior mentioned Scorpion primers and subsequent HRM analysis (see Material and Methods for real-time PCR conditions). Additionally, in every run the plasmids wildtype, 1- and 2-bp deletion served as control for the reliability of the method (not shown in fig. 9). Furthermore, a 4421VT3-Pool consisting of equal units of 50 4421VT3 samples was analysed as reference.

Each sample was passed through the first screening a twice, once with the HRM kit from Qiagen and once with a self-made mastermix containing a Go Taq DNA polymerase and the fluorescent dye SYTO. Thus the two mastermixes could be compared and the results have a higher explanatory value.

4.1.2.1. *Analysis with the HRM-Kit from Qiagen*

Fig. 9 shows the amplification curves of the first screening (the first 50 samples) obtained with Scorpion primers and the corresponding dye, Fluorescein, by using the commercial HRM kit from Qiagen.

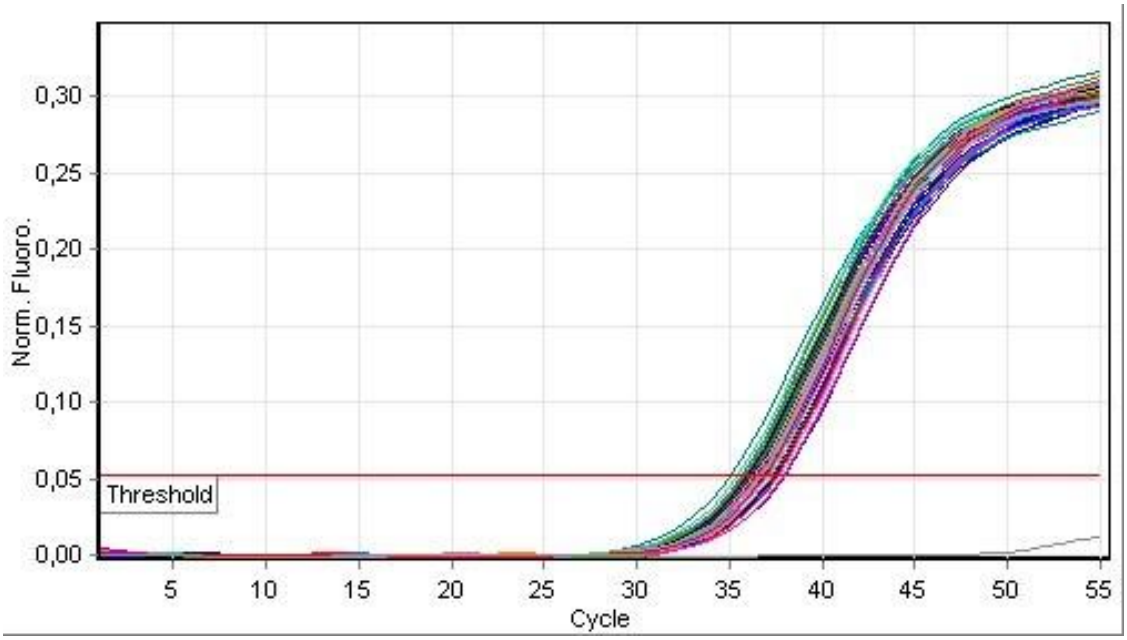

Fig. 9: Illustration of the first screening obtained with Scorpion primers by using the HRM kit from Qiagen

The amplification curves shown in fig. 9 all have the same course and there is no sample with markedly differing amplification curve. This means that there is most likely no sample which has a SNP in the probe. However, this does not mean that no SNPs are present in the target region. To filter out the latter, the obtained Ct values were evaluated in detail. Table 8 shows the Ct values corresponding to the amplification curves from the first screening shown in fig. 9.

No.	Colour	Name	Ct
1		VT3 No. 263	35,67
2		VT3 No. 264	35,04
3		VT3 No. 265	35,99
4		VT3 No. 266	36,20
5		VT3 No. 267	37,28
6		VT3 No. 268	36,20
7		VT3 No. 269	37,27
8		VT3 No. 270	37,51
9		VT3 No. 271	36,55
10		VT3 No. 272	35,81
11		VT3 No. 273	37,96
12		VT3 No. 274	36,23
13		VT3 No. 275	36,02
14		VT3 No. 276	37,28
15		VT3 No. 277	37,35
16		VT3 No. 278	36,47
17		VT3 No. 279	37,62
18		VT3 No. 280	37,01

No.	Colour	Name	Ct
19		VT3 No. 281	36,46
20		VT3 No. 282	36,75
21		VT3 No. 284	36,79
22		VT3 No. 285	37,41
23		VT3 No. 286	36,13
24		VT3 No. 287	35,86
25		VT3 No. 288	36,71
26		VT3 No. 289	36,68
27		VT3 No. 290	35,97
28		VT3 No. 294	35,71
29		VT3 No. 295	36,36
30		VT3 No. 296	35,86
31		VT3 No. 297	35,97
32		VT3 No. 298	36,25
33		VT3 No. 299	37,52
34		VT3 No. 301	36,34
35		VT3 No. 302	36,93
36		VT3 No. 303	36,10

No.	Colour	Name	Ct
37		VT3 No. 304	36,56
38		VT3 No. 305	36,91
39		VT3 No. 306	36,07
40		VT3 No. 307	37,17
41		VT3 No. 308	35,64
42		VT3 No. 315	37,88
43		VT3 No. 316	35,95
44		VT3 No. 318	36,56
45		VT3 No. 319	36,72
46		VT3 No. 320	35,77
47		VT3 No. 321	35,43
48		VT3 No. 322	36,59
49		VT3 No. 323	36,98
50		VT3 No. 324	36,66
51		VT3 Pool	36,89
52		ntc	

Table 8: Ct values obtained from real-time PCR with Scorpion primers using the HRM kit from Qiagen (corresponding to Fig. 9)

The Ct values shown in table 8 range from 35.04 (sample no. 264) to 37.96 (sample no. 273). The 4421 VT3 samples have an average Ct value of 36.54 and a standard deviation of 0.65 Ct units. Accordingly, the variation coefficient is 1.79%.

As with Neumann, et al., it was considered that samples which contain SNPs in the analysed sequence would exhibit comparatively high Ct values. However, this may also indicate a lower DNA concentration [Neumann, et al. 2011]. Con-

sequently, for the evaluation of the screening, the 20% of the samples with the highest Ct values were determined as possible mutants under the condition that they showed a normal curve running with EvaGreen (see fig. 10). The following samples (taken from the first 50) met these criteria: samples no. 269, 267, 276, 277, 285, 270, 299, 279, 315 and 273. Fig. 10 illustrates the amplification plots obtained with EvaGreen during the real-time PCR (Ct values not shown).

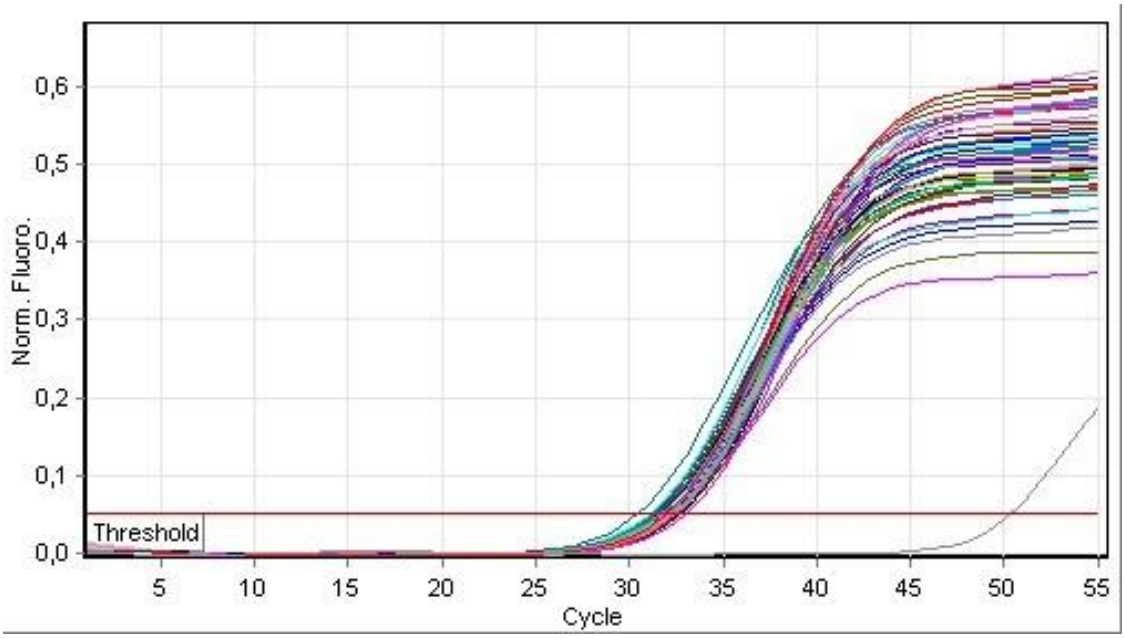

Fig. 10: Illustration of the first screening obtained with EvaGreen by using the HRM kit from Qiagen

As seen in fig. 10, there are some different amplification plots in the upper region. Referring to the theory that DNA samples with lower quality or non GMO samples would result in small or no amplification curves, respectively in very high Ct values [Neumann, et al. 2011] four samples (no. 263, 265, 286 and 287) were excluded.

However, as mentioned before, HRM analysis was used for the evaluation of possible mutants, too. Thus, the consideration of the Ct values alone is not sufficient for a complete evaluation. Also, the confidence values obtained with the HRM analysis must be included. The confidence values of the first screening from the first 50 samples are shown in table 9, while the corresponding melting curves are illustrated in fig. 11 (in normalized form).

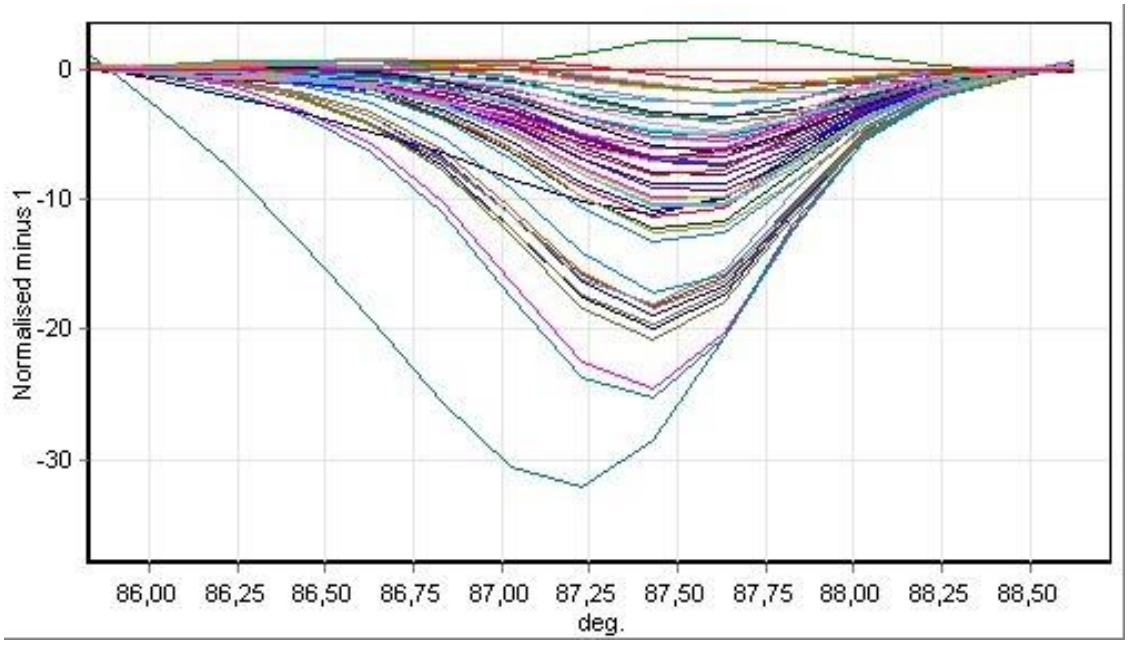

Fig. 11: Normalized difference graph obtained with HRM analysis by using the HRM kit from Qiagen

Figure 11 shows the results of the first screening obtained with HRM analysis by using the HRM kit from Qiagen and using the dye EvaGreen provided in the kit. The horizontal straight line at the zero mark represents the reference against which all other samples were measured. As mentioned above, the 4421 VT3-Pool served as reference. Samples whose normalized melting curves have the largest distance to the reference differ most from the reference. Since the confidence value of the reference is automatically 100%, the samples with the greatest deviation have the smallest confidence values. Table 9 provides an overview of the confidence values obtained with the HRM analysis.

No.	Colour	Name	C. %
1		VT3 No. 263	10,30
2		VT3 No. 264	0,81
3		VT3 No. 265	26,48
4		VT3 No. 266	61,75
5		VT3 No. 267	98,03
6		VT3 No. 268	82,04
7		VT3 No. 269	71,33
8		VT3 No. 270	98,90
9		VT3 No. 271	51,41
10		VT3 No. 272	28,47
11		VT3 No. 273	77,85
12		VT3 No. 274	64,82
13		VT3 No. 275	28,55
14		VT3 No. 276	22,48
15		VT3 No. 277	23,17
16		VT3 No. 278	56,67
17		VT3 No. 279	98,44
18		VT3 No. 280	90,90
19		VT3 No. 281	68,08
20		VT3 No. 282	8,56
21		VT3 No. 284	83,69
22		VT3 No. 285	56,98
23		VT3 No. 286	20,10
24		VT3 No. 287	29,50
25		VT3 No. 288	98,92
26		VT3 No. 289	78,44
27		VT3 No. 290	94,87
28		VT3 No. 294	89,08
29		VT3 No. 295	83,28
30		VT3 No. 296	82,07
31		VT3 No. 297	85,45
32		VT3 No. 298	95,05
33		VT3 No. 299	99,29
34		VT3 No. 301	67,81
35		VT3 No. 302	73,41
36		VT3 No. 303	83,55
37		VT3 No. 304	92,02
38		VT3 No. 305	33,47
39		VT3 No. 306	90,25
40		VT3 No. 307	89,86
41		VT3 No. 308	98,95
42		VT3 No. 315	89,28
43		VT3 No. 316	86,34
44		VT3 No. 318	97,16
45		VT3 No. 319	55,03
46		VT3 No. 320	94,10
47		VT3 No. 321	65,53
48		VT3 No. 322	64,90
49		VT3 No. 323	97,34
50		VT3 No. 324	82,73
51		VT3 Pool	100,00
52		ntc	

Table 9: Confidence values obtained with HRM analysis by using the HRM kit from Qiagen

Table 9 shows the confidence values corresponding to the normalized melting curves illustrated in fig. 11. The confidence values range from 0.81 to 99.29% (without reference). The procedure for the evaluation of the data was basically the same as in the analysis of the Ct values described above. Similarly, 20% of the samples which point most to SNPs in the 3´ MON810 target region were selected. As already described, the samples with the lowest confidence values indicate most SNPs. The following samples were chosen according to the men-

tioned criteria: samples no. 264, 282, 263, 286, 276, 277, 265, 272, 275 and 287.

By comparing the selected samples obtained from the evaluation of the Ct values and the confidence values, two matches were found. Samples no. 276 and 277 suggest in both cases mutations. Thus, these samples were appropriate for the second screening under the condition that the same results were obtained with the self-made mastermix.

The next step was to repeat the first screening with the self-made mastermix.

4.1.2.2. *Analysis with the self-made mastermix*

In the analysis of the self-made mastermix all real-time PCR conditions remained the same, only the reagents were changed. The self-made mastermix contained a Go Taq Hot Start DNA polymerase and the fluorescent dye SYTO next to other chemicals described under Material and Methods.

Fig. 12 shows the amplification curves obtained with real-time PCR by measuring the fluorescent dye SYTO.

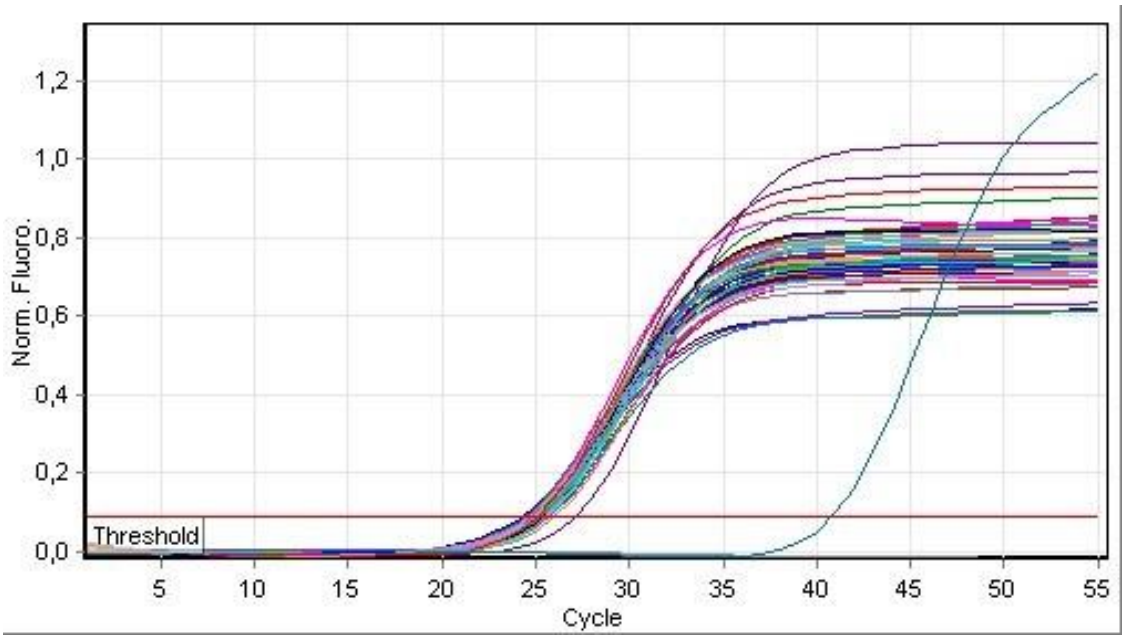

Fig. 12: Illustration of the first screening obtained with SYTO by using the self-made mastermix

As seen in fig. 12 the amplification curves related to SYTO vary relatively strongly compared with the amplification curves obtained with EvaGreen illustrated in fig. 10. Especially, the plateau is quite wide spread in fig. 12 while in the exponential increase only two samples behave highly differently. It was decided that these two samples should not be considered further because they are likely to have bad DNA quality. In summary, five samples (no. 263, 265, 286, 287 and 299) were excluded because of very high Ct values or/and differing amplification curves. Sample no. 305 also indicates low DNA quality but shows a relatively low confidence value (see below). Therefore this sample was not excluded.

The curve, which increases shortly after the 35th cycle, corresponds to the non-template control (NTC). An amplification of the NTC indicates undesired DNA contamination. A disadvantage of SYTO is that this dye is not sequence specific and therefore intercalates with every double stranded DNA molecule. But with subsequent HRM analysis the problem is overcome.

However, normally there should be no amplification but the NTC is clearly distinguishable from the other samples. Therefore it was assumed, that the present run could be used. Fig. 13 illustrates the Scorpion results of the first 50 samples obtained with the self-made mastermix.

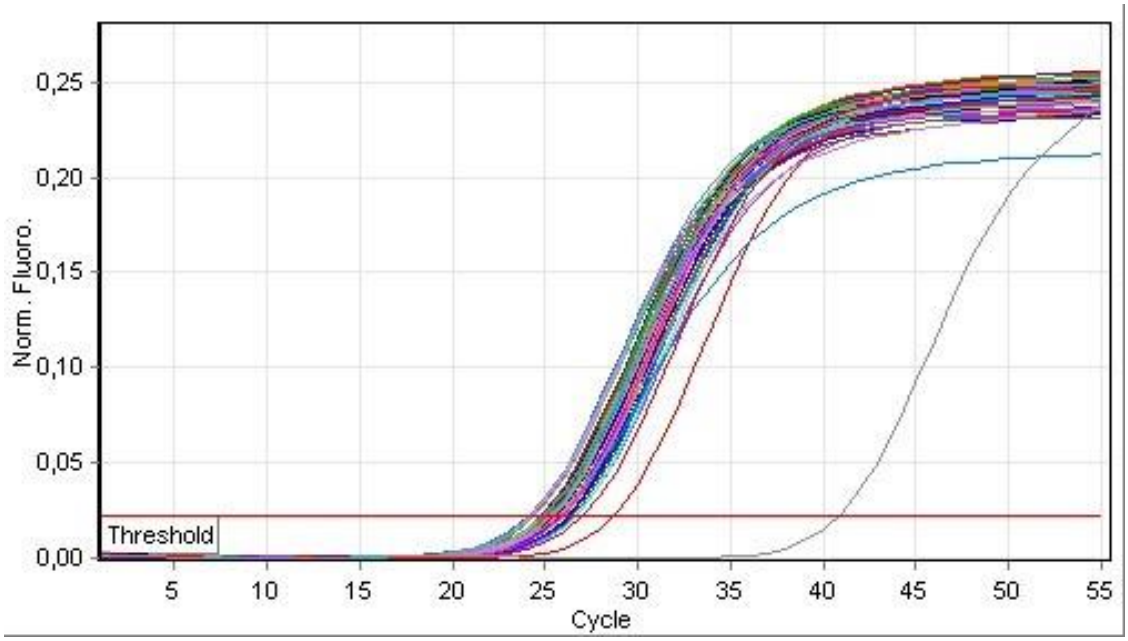

Fig. 13: Illustration of the first screening obtained with Scorpion primers by using the self-made mastermix

As seen and explained in fig. 12, in fig. 13 the NTC also shows amplification. The amplification curves in fig. 13 show a very similar running in all except two samples. This might be due to SNPs in the probe or primer sequence but these samples also have high Ct values with SYTO. Further, the Scorpion analysis with the HRM kit showed no differences with these samples (see fig. 9). Therefore, it was assumed that the differences are referred to bad DNA quality. Table 10 shows the values corresponding to fig. 13.

No.	Colour	Name	Ct
1		VT3 No. 263	24,27
2		VT3 No. 264	24,08
3		VT3 No. 265	24,84
4		VT3 No. 266	24,86
5		VT3 No. 267	26,21
6		VT3 No. 268	24,70
7		VT3 No. 269	25,82
8		VT3 No. 270	26,56
9		VT3 No. 271	24,98
10		VT3 No. 272	25,25
11		VT3 No. 273	26,27
12		VT3 No. 274	25,51
13		VT3 No. 275	24,98
14		VT3 No. 276	25,85
15		VT3 No. 277	25,90
16		VT3 No. 278	25,47
17		VT3 No. 279	25,90
18		VT3 No. 280	25,81

No.	Colour	Name	Ct
19		VT3 No. 281	25,07
20		VT3 No. 282	24,98
21		VT3 No. 284	25,02
22		VT3 No. 285	25,80
23		VT3 No. 286	24,34
24		VT3 No. 287	24,11
25		VT3 No. 288	25,99
26		VT3 No. 289	24,71
27		VT3 No. 290	26,49
28		VT3 No. 294	26,65
29		VT3 No. 295	25,74
30		VT3 No. 296	25,79
31		VT3 No. 297	24,91
32		VT3 No. 298	25,40
33		VT3 No. 299	28,67
34		VT3 No. 301	24,95
35		VT3 No. 302	26,19
36		VT3 No. 303	25,72

No.	Colour	Name	Ct
37		VT3 No. 304	24,26
38		VT3 No. 305	25,71
39		VT3 No. 306	25,35
40		VT3 No. 307	25,58
41		VT3 No. 308	25,17
42		VT3 No. 315	25,87
43		VT3 No. 316	24,85
44		VT3 No. 318	25,26
45		VT3 No. 319	25,39
46		VT3 No. 320	25,01
47		VT3 No. 321	24,38
48		VT3 No. 322	25,27
49		VT3 No. 323	24,85
50		VT3 No. 324	25,73
51		VT3 Pool	27,01
55		ntc	40,82

Table 10: Ct values obtained from real-time PCR with Scorpion primers using the self-made mastermix

The Ct values seen in table 10 range from 24.08 to 28.67 with an average Ct value of 25.44. Further, the calculation of the standard deviation results in 0.82 Ct units and the variation coefficient is 3.2%.

However, the same analysis performed with the HRM kit gives Ct values around 36.54. But with the HRM kit the increase of the curve is approximately after 27 cycles (see fig. 9) while with the self-made mastermix the increase is roughly

after 19 cycles (see fig. 13). Therefore the Ct values are not comparable directly. Samples no. 299, 294, 270, 290, 273, 267, 302, 288, 279 and 277 give the highest Ct values. From these samples no. 267, 277, 270, 299, 279 and 273 agree with the selected samples from the Scorpion analysis with HRM kit. The results obtained with the subsequent HRM analysis are presented in table 11 (normalized difference graph not shown).

No.	Colour	Name	C. %
1		VT3 No. 263	12,44
2		VT3 No. 264	1,00
3		VT3 No. 265	71,21
4		VT3 No. 266	68,14
5		VT3 No. 267	93,76
6		VT3 No. 268	90,05
7		VT3 No. 269	83,78
8		VT3 No. 270	99,22
9		VT3 No. 271	67,85
10		VT3 No. 272	20,14
11		VT3 No. 273	91,46
12		VT3 No. 274	58,02
13		VT3 No. 275	36,99
14		VT3 No. 276	40,46
15		VT3 No. 277	26,05
16		VT3 No. 278	79,18
17		VT3 No. 279	99,38
18		VT3 No. 280	98,16
19		VT3 No. 281	58,71
20		VT3 No. 282	21,18
21		VT3 No. 284	78,13
22		VT3 No. 285	78,98
23		VT3 No. 286	35,72
24		VT3 No. 287	41,48
25		VT3 No. 288	98,16
26		VT3 No. 289	68,67
27		VT3 No. 290	99,66
28		VT3 No. 294	97,58
29		VT3 No. 295	98,24
30		VT3 No. 296	96,88
31		VT3 No. 297	90,10
32		VT3 No. 298	99,20
33		VT3 No. 299	97,22
34		VT3 No. 301	77,63
35		VT3 No. 302	76,20
36		VT3 No. 303	83,81
37		VT3 No. 304	99,48
38		VT3 No. 305	62,82
39		VT3 No. 306	99,36
40		VT3 No. 307	89,20
41		VT3 No. 308	94,91
42		VT3 No. 315	94,42
43		VT3 No. 316	93,64
44		VT3 No. 318	97,22
45		VT3 No. 319	65,47
46		VT3 No. 320	93,13
47		VT3 No. 321	58,04
48		VT3 No. 322	52,56
49		VT3 No. 323	92,15
50		VT3 No. 324	51,16
51		VT3 Pool	100,00

Table 11: Confidence values of the HRM analysis obtained with the self-made mastermix

The Confidence values presented in table 11 range from 1 to 99.66%. Samples no. 264, 263, 272, 282, 277, 286, 275, 276, 287 and 324 have the lowest Confidence values. Except sample no. 324 all selected samples agree with the chosen samples from the HRM analysis with the HRM kit from Qiagen. This high consensus, despite the different mastermixes demonstrates the reliability of the method.

4.1.2.3. *Comparison of the mastermixes*

The results obtained with the two different mastermixes are similar, especially in the case of HRM analysis and DNA quality control with EvaGreen or SYTO. However, the results of Scorpion analysis agree in roughly 60% of cases.

Differences between the mastermixes are the polymerase, the fluorescent dye, the volume of the reaction mixture and the concentration of the different reagents. However, the same primer pair was used as well as the same real-time PCR conditions. Also the DNA polymerases are not very different; both go back to *Thermus aquaticus,* need an activation step and have no proof reading function. Further the fluorescent dyes are very similar, both intercalate into double-stranded DNA and have similar absorbance and emission maxima.

The HRM kit has the advantage that the mastermix is ready for use and therefore saves time. However the self-made mastermix is more favourable from a financial view. It was assumed that both mastermixes are suitable for the anaylsis of SNPs by real-time PCR.

4.1.2.4. *Overview of the first screening*

After screening 100 different 4421VT3 samples two times (only the first set containing 50 samples is presented in detail before) and thereby comparing the two mastermixes it was decided which samples are suitable for the second screening.

Tables 12 and 13 give an overview of the selected samples of the first screening. Table 12 illustrates the results of the first 50 samples while table 13 shows the results of the second 50 samples. The results of the first set are described in detail above, while the results of the second set are not shown in detail.

Selected samples (no.) of the first 50th set

Scorpion a)	Scorpion b)	HRM a)	HRM b)
269	299	264	264
315	294	263	263
270	270	272	272
285	290	282	282
273	273	265	324
267	267	286	286
299	302	275	275
276	288 (276)*	276	276
279	279	287	287
277	277	277	277

(a) Analysis with HRM kit (b) Analysis with self-made mastermix
*sample no. 276 differs only 0.21 units from sample no. 288 and is therefore included
Table 12: Overview of selected samples of the first 50 samples of the first screening

Selected samples (no.) of the second 50th set

Scorpion a)	Scorpion b)	HRM a)	HRM b)
373	327	355	355
363	351	345	345
374	352 (374)*	374	374
356	356	383	383
332	368	347	347
369	369	354	354
372	372	372	333
353	353	352	352
314	314	360	360
361	361	349	334

(a) Analysis with HRM kit (b) Analysis with self-made mastermix
*sample no. 374 differs only 0.03 units from sample no. 352 and is therefore included
Table 13: Overview of selected samples of the second 50 samples of the first screening

Total agreements are highlighted orange in tables 12 and 13.

In the first screening three samples, no. 276, 277 and 374 indicate mutations with Scorpion as well as with HRM analysis. Further, independent from the mastermix, the same results were obtained. These cases are described as total agreements and are highlighted orange in tables 12 and 13. Table 14 summarizes the numbers of selected samples from the first screening.

	Scorpion analysis (a)	**Scorpion analysis (b)**	**HRM analysis (a)**	**HRM analysis (b)**	**DNA control failed (a)**	**DNA control failed (b)**
No. selected samples	20	20	20	20	6	12
Matches (a) and (b)	13		18		6	
Matches Scorpion & HRM	3				----------	

(a) Analysis with HRM kit (b) Analysis with self-made mastermix

A total of 100 samples was tested with the HRM kit as well as with the self-made mastermix in the first screening.

Table 14: Overview of the first screening

Table 14 illustrates a summary of the results of the first screening. In the Scorpion and HRM analysis the number of selected samples is always 20 because it was decided that from every run the 20% of the samples with the highest Ct values and the lowest Confidence values should be selected. Further, in case of Scorpion analysis, the consensus between the two mastermixes is 65%. With HRM analysis the agreement between the two mastermixes is 90%. Six samples failed the DNA control in the evaluation of the fluorescence increase from EvaGreen (corresponding to the HRM kit). The same samples failed the DNA control with the fluorescence dye SYTO (corresponding to the self-made mas-

termix). In addition, with SYTO six more samples showed differing amplification behaviour.

As mentioned before, from the matches of HRM analysis three samples agree with the matches of Scorpion analysis (no. 276, 277 and 374). Thus it was assumed that these three samples should definitely enter the second screening. Sample no. 372 shows not a total, but a partial agreement. Therefore this sample was selected too. Further, one more sample was chosen because of a very low Confidence value (no. 383). In addition, six samples, no. 371, 373, 319, 305 and 352 were already filtered out in a pre-screening (data not shown). Thus, a total of ten samples was cloned into plasmids for the second screening.

4.1.3. Second Screening

In the first screening, the number of false positive and false negative samples were kept low implenting the following measures:

- the measurements of the first screening were repeated on another day for both 50th groups,
- scores of HRM analysis as well as scores of Scorpion analysis were considered,
- a DNA quality control was performed by using SYTO.

Despite these measures some SNPs may be hard to identify in case of heterozygous samples.

Madi et al. (2013) produced homozygous samples with either wildtype or mutated DNA by cloning PCR-DNA of heterozygous alleles into plasmids. This approach simplifies the identification of SNPs in case of heterozygous DNA samples [Madi, et al. 2013].

Therefore the selected samples from the first screening were cloned into plasmids. Because the distribution of wildtype and mutated DNA obtained through cloning is unknown, from each of the selected samples which would undergo

the second screening test at least three plasmids were produced. Thus, the probability of undetected mutated alleles was decreased.

By using the TA cloning kit from Invitrogen the 3´ MON810 sequence illustrated in fig. 6 was cloned into JM109 competent cells from Promega. Blue-white selection was used as selection method to filter colonies which contain the desired insert. Then, white colonies were tested for the correct length of the relevant insert using 2.5% agarose gels. Positive colonies were cultivated in 5ml LB medium. Subsequently, the DNA was isolated with the Easy Prep Pro miniprep kit (Biozym).

Further, the DNA concentration and the degree of purity (ratio: 260nm/280nm) of the purified plasmids were measured with a Nanophotometer^{TM} from IMPLEN. The DNA concentrations of the produced plasmids ranged from 130ng/µl to 398ng/µl with an average of 241ng/µl. To evaluate the purity of DNA the quotient of absorbance at 260nm and 280nm was used. A ratio of ~1.8 indicates highly pure DNA. The presence of proteins, phenols or other reagents is measured at 280nm, therefore the ratio becomes smaller than 1.8 if the DNA extract is impure. In the current study the ratio 260nm/280nm was between 1.8 and 1.9, however, only 5 plasmids had a ratio between 1.9 and 1.97. The average ratio of 260/280 was 1.86 which is in the range for pure DNA [Thermo Fisher Scientific n.d.].

The generated plasmids were diluted to 10^{-4}ng/µl and tested together with the wild type, the 1-bp and the 2-bp plasmid which served as controls. Again, a real-time PCR with Scorpion primers and subsequent HRM analysis was conducted. But for the second screening only the HRM kit was used. The conditions were the same as before. In this way a total of 54 plasmids were generated and analyzed. No plasmid indicated a low DNA quality with EvaGreen. Therefore no plasmids were excluded. Fig. 14 shows the amplification curves obtained with the Scorpion analysis.

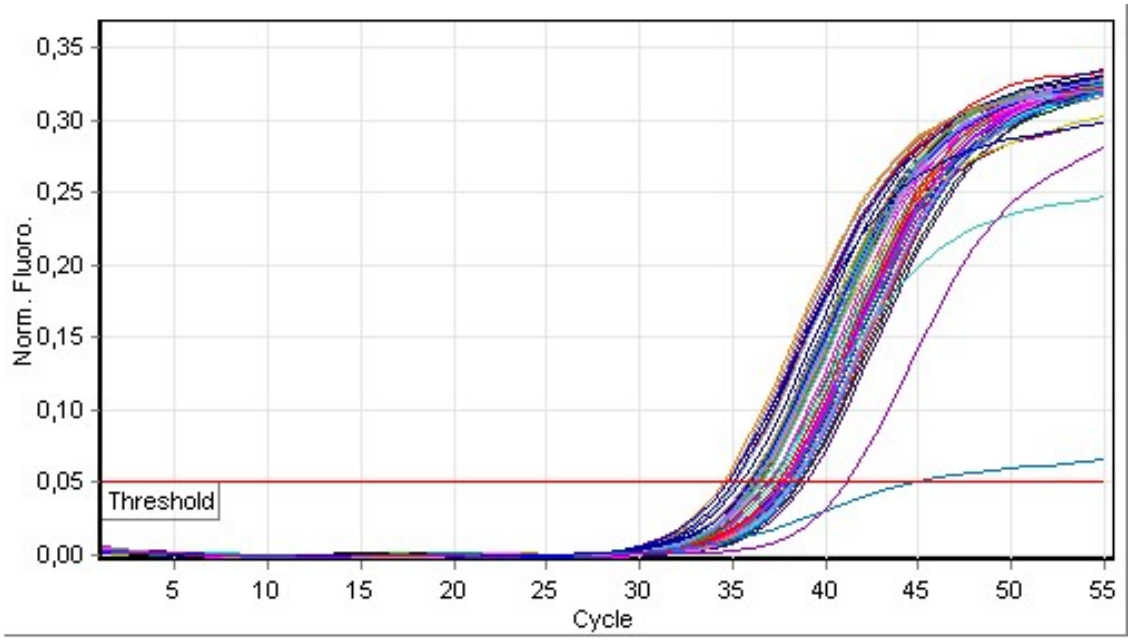

Fig. 14: Second screening with plasmid DNA obtained with Scorpion analysis

As seen in fig. 14 five plasmids differ markedly in their curve profile while the other samples have a similar running. However, for the evaluation the Ct values shown in the following table are necessary.

No.	Colour	Plasmid	Ct
1		No. 373 (1)	36,45
2		No. 373 (2)	36,36
3		No. 373 (3)	38,05
4		No. 373 (4)	35,21
5		No. 373 (5)	34,51
6		No. 373 (6)	35,20
7		No. 374 (1)	37,88
8		No. 374 (2)	37,78
9		No. 374 (3)	35,92
10		No. 374 (4)	36,92
11		No. 374 (5)	38,37
12		No. 374 (6)	38,16
13		No. 374 (7)	35,06
14		No. 374 (8)	35,06
15		No. 374 (9)	35,98
16		No. 319 (1)	38,71
17		No. 319 (2)	36,59
18		No. 319 (3)	38,37
19		No. 319 (4)	36,99
20		No. 319 (5)	37,70
21		No. 305 (1)	36,34
22		No. 305 (2)	37,63
23		No. 305 (3)	37,08
24		No. 305 (5)	37,51
25		No. 371 (1)	37,79
26		No. 371 (2)	37,68
27		No. 371 (3)	37,97
28		No. 371 (4)	37,28
29		No. 372 (2)	38,61
30		No. 372 (3)	37,75
31		No. 372 (4)	37,79
32		No. 372 (5)	38,97
33		No. 372 (6)	36,37
34		No. 383 (1)	36,10
35		No. 383 (2)	35,83
36		No. 383 (3)	34,79
37		No. 383 (4)	36,58
38		No. 352 (1)	38,46
39		No. 352 (2)	36,47
40		No. 352 (3)	36,27
41		No. 352 (4)	36,50
42		No. 352 (5)	36,15
43		No. 352 (6)	36,04
44		No. 276 (1)	36,19
45		No. 276 (2)	35,83
46		No. 276 (3)	36,43
47		No. 276 (4)	36,83
48		No. 276 (5)	36,58
49		No. 277 (1)	36,23
50		No. 277 (2)	38,28
51		No. 277 (3)	37,47
52		No. 277 (4)	36,02
53		No. 277 (5)	35,49
54		No. 277 (6)	36,28

Table 15: Ct values obtained from the second screening with plasmid DNA

Table 15 contains the Ct values obtained from the Scorpion analysis with plasmids. The Ct values range from 34.51 to 38.97 with an average Ct value of 36.83 and a standard deviation of 1.11. Thus the variation coefficient is 3.00%, around the same range as in the first screening.

Fig. 15 shows the difference graph of the plasmid screening obtained with HRM analysis.

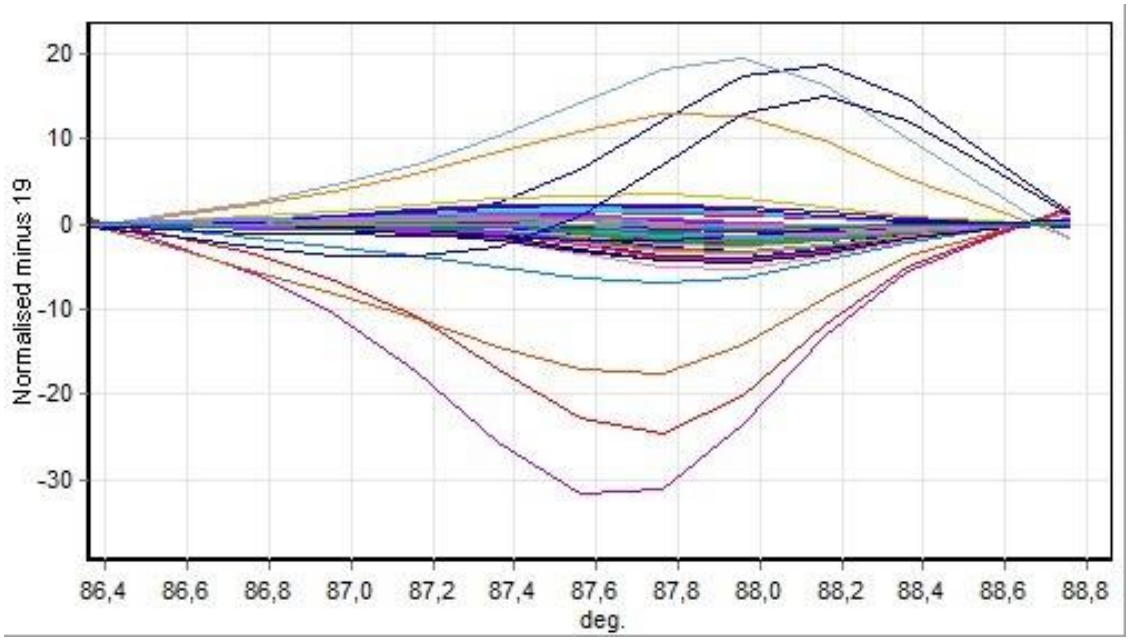

Fig. 15: Second screening with plasmid DNA obtained with HRM analysis by using the HRM kit

In the HRM analysis of the second screening illustrated in fig. 15 the wildtype plasmid mentioned in the pre-test served as reference. In this visualization, seven samples differ markedly from the other plasmids. However, the Confidence values shown in table 16 are more informative for the evaluation of the screening.

No.	Colour	Plasmid	Ct
1		No. 373 (1)	99,36
2		No. 373 (2)	99,76
3		No. 373 (3)	28,36
4		No. 373 (4)	98,07
5		No. 373 (5)	98,03
6		No. 373 (6)	99,56
7		No. 374 (1)	1,67
8		No. 374 (2)	99,14
9		No. 374 (3)	99,01
10		No. 374 (4)	98,39
11		No. 374 (5)	24,81
12		No. 374 (6)	98,75
13		No. 374 (7)	98,61
14		No. 374 (8)	96,73
15		No. 374 (9)	96,59
16		No. 319 (1)	99,16
17		No. 319 (2)	90,56
18		No. 319 (3)	92,85
19		No. 319 (4)	92,61
20		No. 319 (5)	99,14
21		No. 305 (1)	94,72
22		No. 305 (2)	93,44
23		No. 305 (3)	98,67
24		No. 305 (5)	99,70
25		No. 371 (1)	47,51
26		No. 371 (2)	98,99
27		No. 371 (3)	99,38
28		No. 371 (4)	98,52
29		No. 372 (2)	97,27
30		No. 372 (3)	99,30
31		No. 372 (4)	99,55
32		No. 372 (5)	99,82
33		No. 372 (6)	99,60
34		No. 383 (1)	94,44
35		No. 383 (2)	98,13
36		No. 383 (3)	99,72
37		No. 383 (4)	97,74
38		No. 352 (1)	81,06
39		No. 352 (2)	96,06
40		No. 352 (3)	95,95
41		No. 352 (4)	97,95
42		No. 352 (5)	93,88
43		No. 352 (6)	92,63
44		No. 276 (1)	99,08
45		No. 276 (2)	99,68
46		No. 276 (3)	98,47
47		No. 276 (4)	98,96
48		No. 276 (5)	20,81
49		No. 277 (1)	98,26
50		No. 277 (2)	99,23
51		No. 277 (3)	9,75
52		No. 277 (4)	97,72
53		No. 277 (5)	98,10
54		No. 277 (6)	99,18

Table 16: Confidence values of the second screening obtained with HRM analysis

The Confidence values shown in table 16 range from 1.67 to 99.82%. Plasmid samples no. 374(1), 277(3), 276(5), 374(5), 373(3) and 371(1) have the lowest Confidence values (<30%). Therefore these plasmids were selected for sequencing.

Further plasmid samples no. 276(4), 277(5), 373(5) and 383(1) were chosen following a pre-screening with plasmids (data not shown).

Thus from the second screening ten plasmids were selected for sequencing, as they had high Ct or low confidence values, or both. The plasmids refer to six different genomic samples.

4.1.4. Sequencing

Samples no. 276(4), 276(5), 277(3), 277(5), 371(1), 373(3), 373(5), 374(1), 374(5) and 383(1) were determined to be the most suitable for sequencing. But in the end 34 plasmids were sequenced because in case of the presence of a SNP in a plasmid, the sister plasmids were sequenced, too. Sister plasmid means that it is produced from the same genomic sample. However, sister plasmids are generated from different colonies.

Finally, a total of eleven SNPs were identified by sequencing the plasmids forward as well as in reverse except plasmid no. 374 (1) because the reverse sequencing failed. Fig. 16 pictures the identified SNPs in the 3´ T-flanking region.

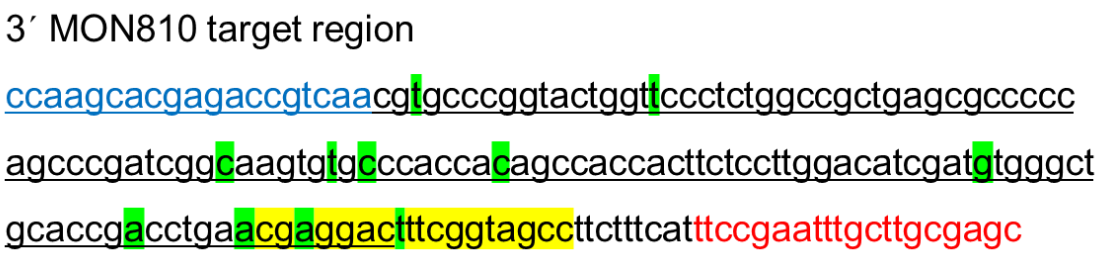

Fig. 16: Documentation of SNPs detected by sequencing plasmids [modified from Neumann, et al. 2011]

Fig. 16 shows the 3´ MON810 target sequence, the forward primer is shown in blue, the reverse primer in red and the probe is marked yellow. The underlined sequence refers to the transgene. In fig. 16 all SNPs detected by sequencing plasmids are highlighted green.

From 5´ end to 3´end following in table 17 presented SNPs are present:

SNP no. [from 5´ end to 3´ end]	**Substitution**	**SNP detected in plasmid no.**	**The plasmid refers to genomic sample no.**	**No. of plasmids generated from the genomic sample**	**No. of plasmids from this genomic sample housing SNPs**
1	t to c	277(5)	277	6	3
2	t to a	373(5)	373	6	2
3	c to t	374(1)	374	9	2
4	t to c	373(3)	373	6	2
5	c to t	277(3)	277	6	3
6	c to t	277(5)	277	6	3
7	g to a	374(5)	374	9	2
8	a to g	276(5)	276	5	2
9	a to g	371(1)	371	4	1
10	a to g	383(1)	383	4	1
11	t to c	276(4)	276	5	2

Table 17: Exemplification of SNPs detected by sequencing plasmids (based on fig. 16)

As illustrated in table 17, from the genomic sample no. 277 six plasmids were generated. From this two plasmids contain SNPs: plasmid no. 277 (3) has one SNP while plasmid no. 277 (5) has two SNPs. Six plasmids were produced from the genomic sample no. 373. Two of this plasmids each have one different SNP. The same with the plasmids generated from the genomic samples no. 374 and 276. Plasmids no. 383 (1) and 371 (1) each house one SNP, too. However, in no case a SNP was found twice.

Plasmid no. 373(5) containing SNP no. 2 (see table 17) was just sequenced because in plasmid no. 373(3) a SNP was detected. The analysis by real-time PCR indicates no SNP. Thus the result of the second screening with plasmid no. 373(5) can be described as a false negative.

No SNPs were detected in the primer regions. SNPs no. 9, 10 and 11 (see table 17) are exactly located in the probe region. SNP no. 9 and 10 are in the transgene while SNP no. 11 lies directly on the first base of the natural genome.

4.1.5. Impact of the detected SNPs

Due to the fact that several SNPs were identified, the impact of the detected SNPs must be clarified. The analysed MON810 sequence encodes partially for the Bt endotoxin of *Bacillus thuringiensis* while the 3´ end of the target sequence belongs to an additional sequence with unknown function merging into the genome. Fig. 17 illustrates the MON810 target sequence with the locations of the detected SNPs again. Additionally, the stop codon is framed in red.

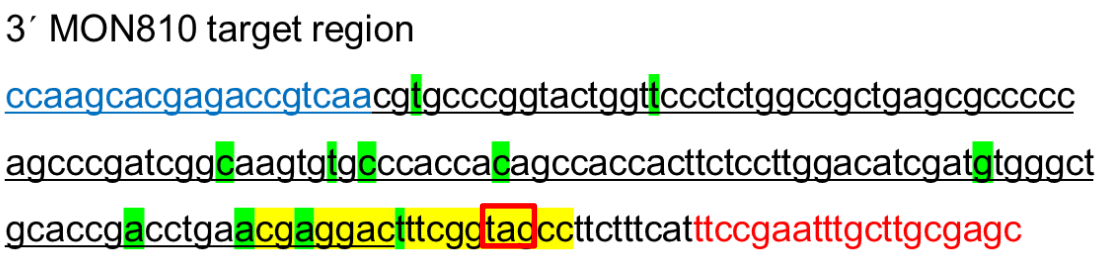

Fig. 17: MON810 target sequence with marked start and stop codons [modified from Neumann, et al. 2011]

The beginning of the target sequence is in the open reading frame (ORF) which ends with the red framed stop codon. Furthermore, the first two letters of the target sequence belong to a codon and so on. The start codon is not in the target sequence. All identified SNPs are located in the ORF.

8 of the identified SNPs cause an amino acid change, 3 SNPs are silent mutations. Silent mutations may also have an impact on plant development.

The present changes would result in a different protein. However, more experiments are necessary to verify the detected SNPs. Fig. 18 gives an overview of the present amino acid changes.

SNP no. [from 5´ end to 3´ end]	**Substitution**	**SNP detected in plasmid no.**	**Amino acid change**	**Type of change**
1	t to c	277(5)	yes	valine → alanine
2	t to a	373(5)	yes	serine → threonine
3	c to t	374(1)	no	-
4	t to c	373(3)	no	-
5	c to t	277(3)	yes	alanine → valine
6	c to t	277(5)	no	-
7	g to a	374(5)	yes	valine → glutamic acid
8	a to g	276(5)	yes	aspartic acid → glycine
9	a to g	371(1)	yes	asparagine → serine
10	a to g	383(1)	yes	glutamic acid → glycine
11	t to c	276(4)	yes	phenylalanine → leucine

Table 18: Amino acid changes

4.2. Results NK603

As already mentioned, the NK603 event could not be verified by the primerpair NK603 NOS-Gen_fwd and NK603 NOS-Gen_rev. The analysis was repeated several times with different 4421VT3 samples. Consequently, other samples (6831RHXT) containing the event NK603 were tested to ensure functionality of the primers. The results of the NK603 check are pictured in fig. 18.

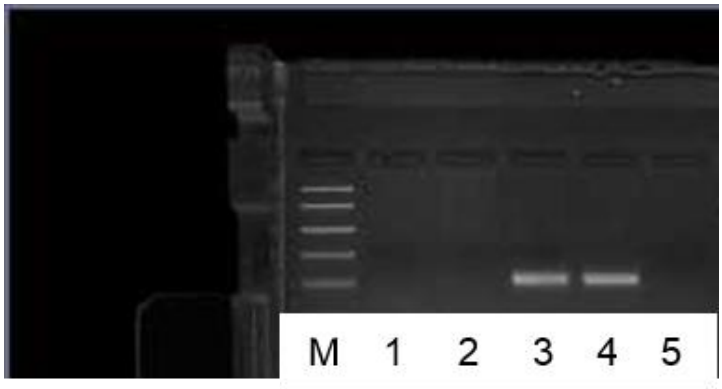

Fig. 18: 2.5% agarose gel showing the evaluation of the NK603 check

Fig. 18 shows the results of the conventional PCR with the primers NK603 NOS-Gen_fwd and NK603 NOS-Gen_rev. M is the marker, from bottom to top the bands correspond to 100, 250, 500, 1000 and 2000bp. No. 1 and 2 belong to 4421VT3 maize while no. 3 and 4 correspond to 6831RHXT maize. No. 5 shows the NTC. Only in 6831RHXT samples the event NK603 could be detected by the mentioned primers. Therefore, it was not clear whether NK603 is included in 4421VT3 maize at all.

Consequently, two other specific tests were conducted with primers located in the area from I-Hsp70 to cp4epsps (see fig. 19). These tests gave negative results for NK603 in 4421VT3 and positive results for NK603 in 6831RHXT too.

Furthermore, two analyzes were performed with primers within I-rAct1 (NK603 Act_fwd1/rev1) and a target sequence ranging from ctp 2 to cp4epsps (see fig. 19). The last mentioned sequence occurs twice in the construct and could be amplified as well as the target sequence located within the section I-rAct1. Thus it was demonstrated that the stacked 4421VT3 maize contains the event NK603. However, the results may indicate a mutation of the event NK603 in 4421VT3 maize. Therefore it was necessary to determine the exact location of the different sections of NK603 in 4421VT3.

4.2.1. NK603 construct

The NK603 construct is composed of twelve sections. Table 19 provides information about the composition of the transgene.

Name	**Description**	**Functions**
****		.
5'FRund	Additional sequence at the 5' flanking region	Not known.
P-ract1	Sequence of the constitutive promoter of the rice (Oryza sativa) actin gene	Constitutive promoter.
I-rAct1	Intron of rice (Oryza sativa) actin 1 gene	Transcription increase intron.
ctp 2	Leader sequence encoding a chloroplast transit peptide from Arabidopsis thaliana	Chloroplast transit peptide.
cp4epsps	Sequence encoding the EPSP enzyme (5-enolpiruvilshikimate- 3-phosphate synthase) from Agrobacterium sp. strain CP4	Glyphosate tolerance.
3' nos	3' termination sequence of the nopaline synthase gene from Agrobacterium tumefaciens T-DNA	Terminator.
P-e35S	Constitutive promoter of CaMV 35S gene	Constitutive promoter.
I-Hsp70	Intron of heat shock protein 70 from Zea mays	intron.
ctp 2	Leader sequence encoding a chloroplast transit peptide from Arabidopsis thaliana	Chloroplast transit peptide.
cp4epsps	Sequence encoding the EPSP enzyme (5-enolpiruvilshikimate- 3-phosphate synthase) from Agrobacterium sp. strain CP4	Glyphosate tolerance.
3' nos	3' termination sequence of the nopaline synthase gene from Agrobacterium tumefaciens T-DNA	Terminator.
3'FRund	Additional sequence at the 3' flanking region	Not known.
****		.

Table 19: Composition of the event maize NK603 [modified from Biosafety Scanner 2013]

As seen in Table 19, two copies of ctp 2, cp4epsps and 3´nos are in the insert. Two different promoters are used, P-ract1 and P-e35S. At the beginning and at the end of the NK603 insert additional sequences with unknown function are present. However, fig. 19 provides more information about the undesired sequence at the 3´ flanking region.

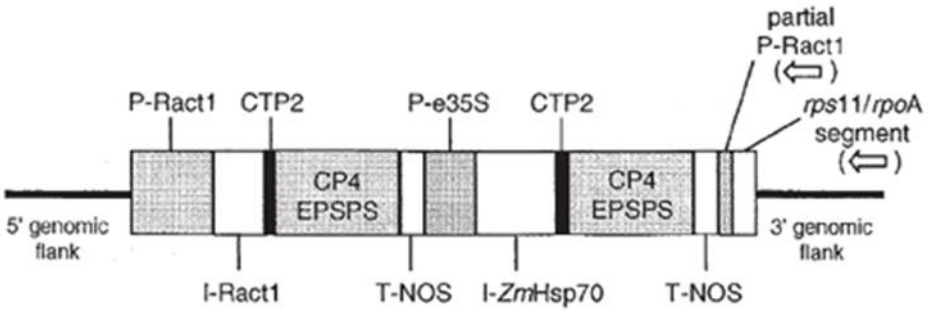

Figure 19: NK603 construct [GMO Detection method Database n.d.]

Figure 19 illustrates the NK603 construct. The pictured parts are described in table 19 except the additional sequence at the 3´ flanking region. Fig. 19 shows that the additional sequence contains parts of the P-ract1 and an rps11/rpoA segment.

Rps11 and rpoA are partial segments of maize plastid genes [Monsanto Company n.d.].

4.2.2. Mapping of the NK603 deletion

For the mapping more primers in different segments were tested. In addition to 4421VT3 maize samples, DNA from 6831RHXT was always used as a control. The aim was to isolate the mutation and clone it into a plasmid for sequencing.

It was obvious that the T-NOS segment at the 3´ flanking region is not intact because no binding of the primers NK603 NOS-Gen_fwd and NK603 NOS-Gen_rev was possible. In contrast the analysis demonstrated that I-rAct1 segment is intact in the main. The same with the transition from ctp 2 to cp4 epsps. However, it was not clear if the amplified sequence was from the ctp 2 to cp4 epsps transition at the 3´ flanking region or 5 ´flanking region or both. Furthermore no amplification was reached between I-Hsp70 and cp4 epsps as well as between I-Hsp70 and ctp 2. Therefore it was assumed that I-Hsp70 might also be affected by a mutation while still it was not clear if segments ctp 2 and cp4 epsps following I-Hsp70 remained intact.

Thus it was decided to work from the 5´end to the 3´end. A 355bp PCR product ranging from I-rAct1 to cp4epsps was amplified successfully in 4421VT3 maize.

However between P-e35S and ctp 2 no amplification was possible in 4421VT3 maize. The same with a sequence ranging from cp4 epsps to the 3´ end.

Hence, the results indicate a mutation ranging from P-e35S at the latest to the 3´ end. This means that the mutation includes almost 3kb. It was questionable if the isolation of such a large deletion sequence may require too much work for the extent of a master thesis.

With a forward primer in section cp4 epsps and a reverse primer at the 3´ end a fragment (regular >3kb) was amplified. However the PCR products were checked on 2.5% agarose gel and subsequently cut out of the gel. Fig. 20 shows the results of the verification.

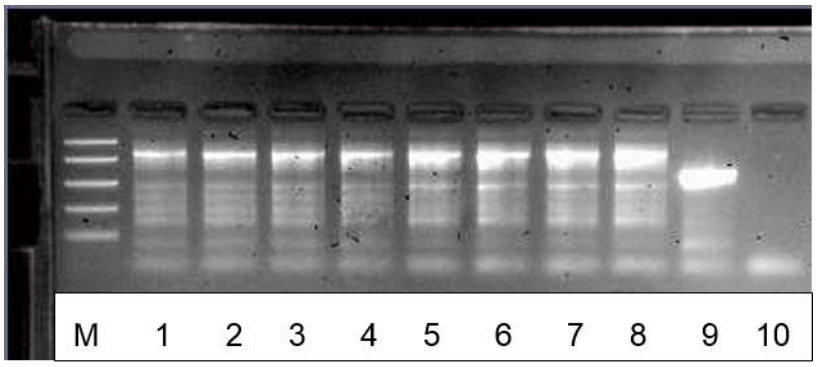

Fig. 20: Verification of PCR products on 2.5 agarose gel linked to the attempt to isolate the deletion

M belongs to the marker. From bottom to top the bands are for 100, 250, 500, 1000 and 2000bp. No. 1 to 8 are the 4421VT3 samples which were cut out of the gel under UV light (for more information see gel extraction under methods) Sample no. 9 refers to the control 6831RHXT while no. 10 is the NTC. After isolating, the bands were purified using Gel Extraction Kit from Omega.

Subsequently a semi-nested PCR was conducted with the purified gel bands. The forward primer was the same as before while the reverse primer was replaced by NK603 NOS-Gen_rev. Afterwards this sequence was cloned into a plasmid and sequenced. Fig. 21 shows the sequencing result of the plasmid.

gttgccggtcttgcgatgataagtaaagctcttcgcaaggcaataccgacagtatccgcttgacctttctaagcg gagacagaatgaaacgaccgtaataaagacgtttactatctactcttgactcaacacacttccactctagtgtttg agtggatcctgttatctctt

Fig. 21: Plasmid sequence of the suspected NK603 deletion

The forward primer is highlighted yellow while the reverse primer is highlighted green. The sequence highlighted green shows the agreement with the NK603 insert. The reverse primer has additionally 15 bases which match with the insert. However, the forward primer sequence is in the plasmid, but only the forward primer matches with the insert. No additional base after the forward primer agrees with the NK603 insert. Therefore it was assumed that the NK603 deletion was not isolated completely because it is implausible that the deletion begins directly after the forward primer.

Subsequently a nucleotide blast search was conducted with the obtained NK603 sequence mentioned above. The search gave 88% query cover with

- Coix lacryma-jobi chloroplast, complete genome,
- Sorghum bicolor mitochondrion, complete genome,
- Zea mays clone EL01N0515A02.d mRNA sequence,
- Zea mays cultivar B73 chloroplast, complete genome,
- Zea mays complete chloroplast genome and
- Zea mays chloroplast rpoA gene for RNA polymerase alpha-subunit.

The identity was 99% in all listed covers above. The 88% query cover was because the sequence of the forward primer did not match with the covers.

Further cloning experiments were performed to isolate the NK603 deletion but with many of the plasmids, sequencing error occurred for unknown reasons. Some plasmids contained sequences of both used primers but no further identity with the NK603 insert. It was considered that the mapping of the NK603 deletion is beyond the scope of a master thesis. Therefore the NK603 experiments were closed at this point. In future, more experiments must be carried out to clarify the exact location and size of the NK603 deletion.

5. Discussion

The genetic stability of the insert of the MON863 x MON810 x NK603 hybrid seed (F1) is assessed as safe by the applicant Monsanto. Since the stacked event is a result of conventional breeding each parental line passes on its single dominant genes to the stacked event by the Mendelian rules. The latter and the genetic stability for the F1 generation have been verified by Monsanto using Southern blot analyses and inheritance pattern studies (Monsanto n.d.) Therefore the requirement that each event of the stacked has the same molecular properties and characteristics as the single events is verified [EFSA 2007].

Monsanto states that the genetic stability is only important for the F1 generation because the F2 generation becomes harvested and marketed. The F2 generation is not intended for further breeding [Monsanto n.d.]. However, DNA changes in the F2 generation are also important as the seeds are used for animal as well as human consumption. Furthermore, it cannot be excluded that farmers use the F2 generation seeds for further breeding although they are not intended for further breeding (e.g. for financial reasons).

Wilson et al. (2006) criticize the current regulatory practice as it is unable to identify and prevent the release of GM crops with potentially harmful genotypic and phenotypic consequences. One critic point of Wilson et al. (2006) are the insufficient analyses of the geno- and phenotype. On the genomic level no genome-wide mutation analysis is required [Wilson, et al. 2006]. Further, the genetic stability is not hedged on the molecular level since Southern blot analysis is considered to be sufficient.

Additionally, if genetic instabilities are verified, there are no guidelines how to handle with the data in detail. Although Rosati et al. (2008) demonstrated the expression of new proteins giving no homology with any known proteins, the EFSA considered that there must not be any concerns regarding the newly expressed proteins or the genetic stability without giving a reference to the statement [EFSA 2009, Rosati, et al. 2008]. One reason for the insufficient verifica-

tion might be that there are no specific guidelines for special scenarios to categorize events into potentially harmful or not harmful. Thus the regulators have no detailed instructions on how to handle with new data [Wilson, et al. 2006].

The requirement for GMO monitoring is generally accepted but its implentation concerning scope and objectives as well as monitoring parameters is highly discussed between scientists, regulators, applicants and other stakeholders. A more unified solution with standardized methods such as the European CEN Standards and OECD-methods is sought by Germany, Switzerland and Austria. Generally, data submitted by the notifier during the ERA should agree with the current state of the art of scientific knowledge. Thus the data would be more comparable and of higher quality [Environmental Agency 2011].

Due to the present findings it has to be clarified, whether the mutation rate of GMOs or stacked events is increased. If it is increased, it must be analysed whether the mutations are present in the whole genome or just in the transgene and the flanking regions. At the current state it is assumed that mutation rates are different throughout the plant genome. Additionally, maize mutations are not only loci-dependent but also context dependent [Neumann, et al. 2009]. If the T-DNA and the surrounding DNA is more susceptible to mutations as Papazova et al. mentioned [Papazova, et al. 2008], the reasons have to be clarified. Kohli et al. (1999) as well as Ho et al. (1999) refer to the CaMV 35S promoter as possible cause of instabilities. Earlier studies demonstrated that the CaMV RNA promotes viral recombination in dicot plants [Ho, et al. 1999, Kohli, et al. 1999]. Since stacked events contain multiple promoters the susceptibility to instabilities may be increased. The object of the present investigation contains two 35S promoters.

The aim of this project was to check the GM maize 4421VT3 for SNPs. Neumann et al. (2009) demonstrated that Scorpion primers are well suited for such an approach [Neumann, et al. 2009]. Further, Madi et al. (2013) showed that HRM analysis is also an appropriate technique for SNP detection [Madi, et al.

2013]. The idea was to combine Scorpion analysis and HRM analysis for SNP detection in GMOs as an additional approach next to Southern analysis. Thus, the molecular level would also be covered. Sequencing and PCR analyses alone would give an inadequate analysis of the transgene organization as some major rearrangements might not be identified. It should be mentioned that Southern analysis is a suitable tool for the detection of major changes. However it is not suitable for the detection of SNPs [Kohli, et al. 2003]. But SNPs are more common than major mutations [Madi, et al. 2013]. The effects of SNPs should not be underestimated in the assessment of the genetic stability. A SNP in the right spot can decide on survival or extinction of an organism in extreme cases.
However, often they only have an influence on the phenotype [Madi, et al. 2013].

Scorpion primers are favoured over TaqMan probes as the probe reaction is unimolecular because the probe is connected with the PCR primer. Furthermore, Scorpion primers have a high signal-to-noise ratio. They are very sensitive to even minor changes in the target sequence. Even heterozygous alterations can be identified. However, one disadvantage is that a Scorpion analysis cannot include much more than 90 nucleotides. Larger primers lower the sensitivity of the system. Therefore only parts of the constructs can be analysed. However, the experiments of Neumann et al. (2009 & 2011) as well as the present experiments confirm the fitness of Scorpion primers for SNP detection [Neumann, et al. 2009 & 2011, Madi, et al. 2013].

With HRM analysis more than 500 nucleotides can be covered in one analysis. However, in case of potential positive samples it is difficult to locate the nucleotide changes. HRM analysis is favoured for this analysis because it can be performed without knowledge of SNP presence or exact SNP location in contrast to other methods such as second generation sequencing or digestion with restriction enzymes, etc. HRM of PCR products has a long history of use in bio-

medical research concerning SNPs. And it is already proven for SNP detection in plant genomes [Lehmensiek, et al. 2008, Madi, et al. 2013].

It can be assumed that the present combination of Scorpion and HRM scores for the screening of GMOs has many advantages over other methods. The dual approach is very effective in detecting genetic instabilities and has reliable output. Additionally a large number of samples can be analysed performing one PCR run. All laboratories performing GMO investigations already have real-time PCR equipment and are usually familiar with the procedure. Therefore no major purchases must be made. Further, the required protocols are not complicated [Neumann, et al. 2009, Madi, et al. 2013].

The results obtained with plasmids containing the MON810 target sequence indicate that real-time PCR with Scorpion primers is more sensitive in detecting SNPs than the subsequently performed HRM analysis. But to make reliable statements about the sensitivity, further analyzes with plasmids are necessary. However, it must be considered that the HRM analysis is able to detect SNPs in the entire target sequence while the Scorpion analysis can only discover SNPs in the probe and primer region.

Further, HRM analysis has the advantage that it requires only little information in addition to the performance of a real-time PCR. HRM analysis can be accomplished with almost every PCR primer used in conventional PCR while the development of Scorpion primers is complicated and requires more financial resources. Special software is needed and often it is difficult to get appropriate primers for the target region. Madi, et al. demonstrated that in addition to the Scorpion system the HRM analysis is also able to differentiate between different alleles in case of heterozygous DNA samples [Madi, et al. 2013].

The HRM analysis is very meaningful when deviating amplification curves for the Scorpion evaluation occur as well as for the SYTO or EvaGreen check. This may be the case with poor DNA quality, but also with larger mutations. Normal-

ly, in case of poor DNA quality of a sample, the HRM analysis shows no difference in the melting curve and therefore no low confidence value. But if there is a major mutation, the HRM analysis would result in a different melting curve with a low confidence value.

Nevertheless, the Scorpion method has fewer influencing variables. It makes sense to combine real-time PCR with Scorpion primers and HRM analysis which results in a highly suitable method to screen samples for DNA changes [Madi, et al. 2013].

The ability to differentiate between different alleles is a very important property. It is the main reason to use this combined method before sequencing. Sanger sequencing alone is not able to reliably detect heterozygous SNPs. Therefore labor-intensive cloning is performed prior to sequencing. Without using the screening methods Scorpion and HRM analysis it is not clear which samples might harbor mutations. Thus, all samples would have to be cloned into plasmids meaning an unacceptable effort apart from the costs. By using the screening only potential positive samples are cloned into plasmids and subsequently sequenced.

With the awareness that other regions in the genome may be equally affected by mutations, the transitions from the transgene to the genome were selected for the analysis. However, mutations in the flanking regions might have greater consequences as they can cause false negative results in official GMO detection and quantification by PCR methods. Further, most mutations detected in GMOs are located in or around the insert [Neumann, et al. 2009].

Maize grains are an ideal investigation object as they have a high DNA concentration with a low fragmentation level. DNA impurities as well as DNA fragmentations can disrupt the PCR reaction and their inclusion has be avoided [Neumann, et al. 2011].

One more reason for the performed experiments is the fact that independent research is rare in the field of GMOs although it is the most important source of risk assessment.

Several unknown SNPs were identified in the short MON810 target sequence in 4421VT3 maize. This indicates a highly increased mutation rate.
SNPs can be caused by polymerase errors as well as by environmental factors. Small mutations in the gametes can be given to the progenies if they occur prior to fertilization. Plants do not have predetermined germs like animals. Thus somatic mutations can be carried over to the progenies. Such scenarios would be identifiable before their phenotypic expression with the presented experiments. This highlights the meaning of the performed project [Neumann, et al. 2009].

However, none of the identified SNPs occurred in a homozygote status. Prior to the experiments it was assumed that the present maize object is diploid. The results indicate that this is at least partially not true. Some samples have 3 different alleles while in diploid samples a maximum of 2 alleles occur. The only appropriate explanation for up to 3 alleles is that either the maize is polyploid or the SNPs are artefacts.

The latter is not impossible, since the polymerase used has no proof-reading function. On the other hand the same polymerase was already used for the experiments of Madi et al. (2013) where several hundred soybeans as well as maize grains were screened. Only a few samples indicate mutations and sequencing demonstrated no SNPs [Madi, et al. 2013]. Thus, if the fidelity rate of the used polymerase is very low, Madi et al. (2013) would have found SNPs, too. Furthermore, Madi et al. repeated my present cloning experiments with the genomic sample no. 383 by use of an Amplitaq Gold polymerase which has a proof-reading function (data not published). They produced 6 plasmids of sample no. 383 which were sequenced. In four of them the same SNP as in plasmid no. 374(1) was identified (see SNP no.3 in table 18). This indicates that the present SNPs are no ore not only artefacts. Sequencing errors are also excluded

since the samples were sequenced twice (forward and reverse) except plasmid no. 374(1) as mentioned above. However, the SNP of plasmid no. 374(1) was verified by Madi et al (data not published).

Eckert and Kunkel (1990) demonstrated that the thermostable *Thermus aquaticus* (Taq) DNA polymerase can have a very high fidelity although it has no proof reading function. Error rates of the exonuclease-deficient Taq DNA polymerase during PCR vary between 2×10^{-4} and $< 1 \times 10^{-5}$ per nucleotide per cycle. The frequency of polymerase errors depends on the pH value, $MgCl_2$ and dNTP concentration. Equimolar concentrations of $MgCl_2$ and dNTP as well as low pH values result in higher fidelity of the polymerase. However, ideal fidelity conditions do not agree with optimal PCR conditions. In the experiments of Eckert and Kunkel (1990) T → C substitutions were most frequent (56%) under high error conditions [Eckert & Kunkel 1990].

In the present experiments 27% of the detected SNPs were T → C substitutions. It cannot be excluded that some of the detected SNPs refer to polymerase errors. Furthermore, at no time during this project the same SNP was detected twice. Only the SNP no. 3 (see table 18) verified by Madi et al. can be considered reliable as they used a polymerase with a 3'-5' proofreading exonuclease and additionally identified the same SNP 4 times. As seen in table 18, the SNP no. 3 does not lead to a different amino acid pattern in contrast to other SNP findings.

As already mentioned polyploidy can also be an explanation for the identified SNP pattern as polyploidy is very common in plants. Even maize has polyploidy ancestors. Polyploidy means that organisms carry more than two complete sets of chromosomes in their cells. Sexual polyploidization through gametes without reduced chromosome number (2n gametes) is known as the main reason for polyploidization. Normally, 2n gametes occur through the expression of mutations affecting micro- and megasporogenesis [Aversano, et al. 2012].

Triploid as well as tetraploid variants can be formed through crossing two diploid species. Spontaneous triploids are not rare in diploid populations. In many taxa small numbers of polyploids in normally diploid populations can be identified. Also hybrid triploids or tetraploids can occur. They are formed by diploids in the F1 or F2 generation of interspecific crosses. Ramsey and Schemske (1998) analysed the frequency of 2n gametes. The result was that 2n gametes occur 50 times more frequently in hybrids (27.52%) than in nonhybrids (0.56%). The difference was significant. Environmental factors such as temperature influence the frequency of unreduced gametes [Ramsey & Schemske 1998].

The theory of spontaneous polyploidy came about because some analysed samples had 3 different alleles (wildtype and two different SNPs). However, more research is necessary to clarify whether some of the present SNP findings belong to artefacts or a polyploidy status with different alleles. For future investigations it would be important to focus more on the fidelity of the polymerase used.

The investigations of the event NK603 in 4421VT3 maize went differently than planned. Since the conventional PCR analysis indicates a larger deletion in the genome, the mapping of the deletion was started instead of the planned screening by real-time PCR. However, the mapping of the deletion became a challenge. The isolation of the deletion has not yet been fully successful. It is unknown, why some cloned plasmids contained the used primer sequences but no or only one-sided agreement with the NK603 insert. Further, it remains unclear why sequencing errors occurred frequently in NK603 plasmids but not in MON810 plasmids. Sequencing analysis indicate overlays. Therefore the PCR conditions might be not ideal for subsequent sequencing. However, the cloned PCR fragments include ~3kb. For future investigations it would be appropriate to adjust the cloning experiments to the size of the cloned fragments.

It was assumed that the deletion is located between P-e35S at the latest and the 3´ end. As already mentioned the 35S promoter is already known to pro-

mote rearrangements in plants [Kohli, et al. 1999]. Therefore the supposed location of the deletion is not surprising. Normally, such a deletion would have been observed by Southern blotting. But according to Monsanto only the F1 generation of stacked events needs to be analysed by Southern blotting. The generation of the examined seeds is not known. Thus no further statements in this context are possible. But generally only the F1 generation is thought to be used for breeding.

The investigation of the triple-stacked hybrid GM maize 4421VT3 indicates genetic instabilities in contrast to the findings of the applicant. The results of this project confirm the critics of the current regulatory practice by Wilson et al. (2006) [Wilson, et al. 2006]. Further studies are necessary to verify the genetic stability of 4421VT3 maize. Additionally, the results indicate that stacked events are more vulnerable to mutations than single GM events. This might be due to multiple promoters. In any case this should be clarified by future investigations.

Furthermore the mutated areas are not suitable for the official food examination for detection and quantification of GMOs. In case of stacked events the detection and quantification is problematic anyway.
It could be shown that Southern Blot analysis alone is not an adequate method for the control of the genetic stability of GMOs because the method cannot detect small DNA changes. Scorpion primers and HRM analysis in combination are suitable systems to detect possible DNA mutations. However, the methods need to be optimized further. Nevertheless, it should be considered to use them for future security checks of GMOs.

It is necessary to clarify the effects of the detected amino acid changes (after verifying the SNPs), as these lines are planted in the USA and are already allowed for human consumption in many countries. In consideration of the fact that more stacked events are generated with increasing event content, the suspicion of an increased mutation rate should be investigated urgently to prevent ecological disadvantages. Given the potential impact of genomic instabilities on

the plant and the scarcity studies focusing on this topic in relation to GMOs more investigations especially on the molecular level are necessary [Neumann, et al. 2011].

6. Summary

6.1. Abstract (English version)

Since the introduction of GMOs in the EU in 1997 their use has drastically expanded. For the official control and authorization of food, feed and seeds it is therefore necessary to identify and quantify the increasing number of GMO events. In recent years - in addition to the detection of GMOs – it has also become a challenge to investigate the genetic stability of GM plants. According to EFSA and EU Directive 2001/18/EC, it is necessary that the introduced DNA construct in the GMO is stable.

However, to control the genetic stability, at each GMO event few samples are analysed, and only by Southern Blots. This method is not suitable for SNP detection. In this study, a high number of transgenic maize seeds (individual samples) were investigated using methods that allow to detect even small DNA rearrangements and subsequently provide additional information to the prescribed Southern Blot analyses.

The experiments were carried out on the triple-stacked GM maize variety 4421VT3 (MON863 x MON810 x NK603). Two methods were utilised: Real-time PCR with Scorpion primers and HRM analysis. Furthermore, conventional PCR was used for the mapping of a deletion detected in NK603.

The study examined the transitions from the construct into the genome, since they are also used for the quantification of GMOs. With HRM analysis and Scorpion primers this study aimed to check whether a distinction between wild-type and mutation is possible. From 100 individual seed grains DNA was extracted followed by a screening of selected regions using Scorpion and HRM analyses to identify DNA changes. Potentially positive samples were sequenced.

In the events MON810 and NK603 unpublished mutations were identified. A total of eleven SNPs was identified in MON810. In NK603 a large deletion ranging from the 35S promoter to the 3´end of the construct was discovered. The results demonstrate genetic instability in the analysed triple stacked event. The results have to be clarified since the maize variety 4421VT3 is already allowed for planting and consumption in the USA.

6.2. Abstract (German version)

Seit der Einführung von gentechnisch veränderten Organismen (GVO´s) im Jahr 1997 sind GVO´s drastisch expandiert. Für die offizielle Kontrolle und Authorisierung von Lebensmitteln, Futtermitteln und Saatgut muss daher eine steigende Anzahl von GVO Events identifiziert sowie quantifiziert werden. In den letzten Jahren ist es neben der Detektion von GVO auch eine Herausforderung geworden, die genetische Stabilität von GVO Pflanzen zu untersuchen.

Laut EFSA sowie der Richtlinie 2001/18/EC ist es notwendig, dass das transgene Konstrukt im GVO stabil ist. Für diese Kontrollen werden allerdings bei jedem GVO Event nur wenige Proben mittels Southern Blots analysiert, welche nicht für die Detektion von SNPs geeignet sind.

In dieser Studie wurde eine hohe Anzahl transgener Maiskörner (individuelle Proben) mit Methoden analysiert, die auch kleinste DNA Änderungen erkennen können und daher die vorher beschriebenen Southern Blot Analysen ergänzen können. Die Experimente wurden an der dreifach-gestapelten GV-Mais Varietät 4421VT3 (MON863 x MON810 x NK603) durchgeführt.

Zwei Methoden wurden angewandt: Real-time PCR mit Scorpion Primern und HRM Analyse. Zusätzlich wurde die konventionelle PCR für die Kartierung einer in NK603 detektierten Deletion verwendet. In dieser Studie wurden die Übergänge vom Konstrukt ins Genom untersucht, da diese auch für die Quantifizierung von GVO´s verwendet werden. Mittels HRM Analyse und Scorpion Primern wurde überprüft, ob eine Unterscheidung zwischen Wildtyp und Mutant möglich ist. Von 100 einzelnen Körnern wurde DNA extrahiert und anschließend ein Screening ausgewählter Regionen mittels Scorpion und HRM Analysen durchgeführt, um DNA Änderungen zu identifizieren. Potentiell positive Proben wurden sequenziert.

In den Events MON810 und NK603 wurden nicht-publizierte Mutationen identifiziert. 11 SNPs wurden insgesamt in MON810 identifiziert. In NK603 wurde eine große Deletion, die vom 35S Promoter bis zum 3´Ende des Konstrukts reicht, entdeckt. Die Ergebnisse demonstrieren genetische Instabilität in dem analysierten dreifach-gestapelten Event. Da die Mais Varietät 4421VT3 in den USA bereits angepflanzt und konsumiert wird, sollten die Ergebnisse geklärt werden.

7. Appendix

7.1. Literature index

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7.2. Confirmation

Erklärung

Hiermit erkläre ich, Sina-Elisabeth Ben Ali, die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegeben Quellen benutzt zu haben. Des Weiteren versichere ich, die Stellen im Text gekennzeichnet zu haben, die dem Wortlaut oder Sinn nach aus anderen Arbeiten entnommen wurden.

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