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DEVELOPMENT OF QCM BASED DNA BIOSENSORS FOR DETECTION OF GENETICALLY MODIFIED ORGANISMS

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ABSTRACT

DEVELOPMENT OF LABEL FREE DETECTION METHODS FOR GENETICALLY MODIFIED ORGANISMS

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A great effort has been recently devoted to the development of new devices for the detection of specific sequences of DNA, due to increasing need of label - free, fast, cheap, and miniaturized analytical systems able to detect target sequences for screening purposes, especially in food industry for genetically modified organisms (GMOs).

In this study, development of a QCM - based DNA biosensor for the detection of the hybridisation of CaMV 35S promoter sequence (P35S) was investigated. Attention was focused on the choice of the coating chemistry that could be used for the immobilisation of probe sequences on the gold surface of the quartz crystal. Two immobilisation procedures were tested and compared considering the amount of the immobilised probe, the extent of the hybridisation reaction, the possibility of regeneration and the absence of non - specific adsorption.

The two coating methods were based on the use of self - assembled monolayers. One of them employed the interaction between the thiol and gold for the immobilisation of a thiolated P35S probe, while the other employed formation of functionalised aldehyde groups by ethylenediamine plasma polymerization on the gold surface for the immobilisation of amined P35S probes through gluteraldehyde activation. Results indicated that immobilisation of a thiolated probe provides better immobilisation characteristic, higher sensitivity for the detection of the hybridisation reaction, absence of non - specific adsorption and a higher stability with respect to the regeneration step.

The optimised immobilisation procedure for the thiolated probe was used for the detection of P35S sequence in PCR - amplified DNAs and in real samples of *pflp* - gene inserted tobacco plants that produce ferrodoxin like protein additionally. Fragmentation of the genomic DNAs were achieved by digestion with restriction endonucleases and sonication. The obtained results from the fragmented genomic DNAs demonstrated that it is possible to detect the target sequence directly in non-amplified genomic DNAs by using the developed QCM - based DNA biosensor system.

The developed QCM-based DNA biosensor represented promising results for a real-time, label - free, direct detection of DNA samples for the screening of GMOs.

Key words: DNA - based biosensors; Genetically Modified Organisms (GMOs); CaMV P35S; immobilisation

ÖZ

GENETİĞİ DEĞİŞTİRİLMİŞ ORGANİZMALAR İÇİN İŞARETSİZ SAPTAMA METODLARININ GELİŞTİRİLMESİ

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Gıda sektöründe, özellikle genetiği değiştirilmiş organizmalarda (GDO) hedef bölgelerin taranmasını sağlayan hızlı, ucuz ve minyatürize edilmiş analitik sistemlere ihtiyaç duyulmaktadır ve bu nedenle son zamanlarda, spesifik DNA sekanslarını saptayan yeni aygıtların geliştirilmesi için büyük bir çaba harcanmaktadır.

Bu çalışmada, CaMV 35S promotör sekansının hibridizasyon reaksiyonunu saptayan QCM tabanlı bir DNA biyosensörü geliştirilmeye çalışılmıştır. Prob oligonükleotidlerinin kuvartz kristalin altın yüzeyine bağlanması için gerekli olan yüzey kaplama metodunun seçimi için çalışmalar yapılmıştır. İki kaplama metodu denenmiş ve bunlar yüzeye bağlanan probun miktarı, hibridizasyona etkisi, rejenerasyon kapasitesi ve hedefe özgül olma özellikleri bakımından incelenmiştir.

İki kaplama metodu da kendi halinde oluşan tek yüzeylerin kullanımı esasına bağlıdır. Biri, tiol grubu taşıyan P35S prob oligonükleotidin yüzeye bağlanması için, tiol ve altının etkileşimini içerir. Diğeri ise, amin grubu taşıyan oligonükleotidin altın

yüzeye bağlanması için yüzeyin aldehit grupları ile fonksiyonel hale getirilmesini içerir. Sensör yüzeyinin fonksiyonelleştirilmesi, plazma polimerizasyonu yöntemi kullanılarak yüzeye kaplanan etilendiamin monomerinin gluteraldehit ile aktifleştirilmesi yoluyla sağlanmıştır.

Elde edilen sonuçlar, tiol grubu ile yüzeye kaplama yapılan probların daha iyi kaplama özellikleri gösterdiğini, hibridizasyon reaksiyonu sırasında daha yüksek hassasiyet sağladığını, özgül olmayan bağlanma yapmadığı ve rejenerasyon basamaklarına karşı daha dayanıklı olduğunu göstermiştir.

Tiol prob için optimize edilmiş kaplama yöntemi, PZR ile çoğaltılmış ve *pflp* geni aktarılmış tütün bitkisi DNAsında P35S gen dizisinin saptanması amacıyla kullanılmıştır. Kromozomal DNAların parçalanması, kesici enzimler kullanılarak ya da yüksek ses dalgaları yardımı ile sağlanmıştır. DNA örnekleri ile yapılan çalışmalar, geliştirilen QCM bazlı DNA biyosensörünün PZR ile çoğaltılmamış DNA örneklerinde hedef gen dizisinin direkt olarak saptadığını göstermiştir.

Elde edilen sonuçlar, geliştirilen QCM bazlı DNA biyosensörünün, genetiği değiştirilmiş organizmaların saptanması amacıyla kullanılabilecek gerçek zamanlı, işaretsiz ve direkt bir sistem olabileceği yönünde ümit vaad etmektedir.

Anahtar sözcükler: DNA-bazlı biyosensörler; Genetiği değiştirilmiş organizmalar (GDO); CaMV P35S; immobilizasyon

TO MY FAMILY ...

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LIST OF ABBREVIATIONS

CTAB	Hexadecyltrimethylammonium bromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EB	Ethidium bromide
EDA	Ethylene diamine
EDTA	Ethylenediaminetetraacetic acid
GA	Glutaraldehyde
GFP	Green fluorescence protein
GMO	Genetically modified organism
LB	Luria broth
MCH	6-mercapto-1-hexanol
MREs	Molecular recognition elements
P	Promoter
PCR	Polymerase chain reaction
PNAs	Peptide nucleic acids
PPFs	Plasma polymerized films
QCM	Quartz crystal microbalance
RE	Restriction enzyme
RNA	Ribonucleic acid
RR	Round-up ready
SDS	Sodium dodecyl sulfate
SPR	Surface plasmon resonance
ssDNA	Single stranded deoxyribonucleic acid
T	Terminator
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride

CHAPTER I

INTRODUCTION

1.1. Genetically Modified Organisms (GMOs)

For thousands of years, unaware of even the existence of nucleic acids, farmers and plant breeders have been performing an effective form of gene transferring and selection, a process called genetic “manipulation” or “modification”. Commonly crossing and saving seeds of the strongest or most fruitful plants enabled to slowly maintain or improve the most desired characteristics for farming. As all crop and domesticated animal species have undergone human selection since the dawn of time, they can all be considered ‘‘genetically modified’’ with respect to the wild types, even if old farmers did not know they were selecting genes (Paparini and Romano-Spica, 2004).

With the advent of modern biotechnology, specifically “recombinant DNA technology”, it is now possible to transfer a specific gene, called a “transgene”, between species belonging to different genera, families or kingdoms, through a process called “gene transformation” in a much faster and more precise fashion than ever (Viljoen, 2005). Combining genes from different organisms is known as recombinant DNA technology, and the resulting organism is said to be "genetically modified" or "transgenic".

A genetically modified organism (GMO) is referred to a living organism whose genome has been modified by the introduction of an exogenous gene able to express an additional recombinant protein from prokaryotic or eukaryotic organisms in a temporal and spatial manner that confers new characteristics, i.e. herbicide tolerance (De Block *et al.*, 1987) or resistance to virus (Hails, 2000), antibiotic (Dröge *et al.,* 1998) and insect (Vollenhofer *et al.,* 1999; Hails, 2000; Mariotti *et al.,* 2002), for a variety of purposes including agronomic productivity, consumer benefits, and antibody and vaccine development (Mason *et al.,* 2002; Stewart *et al.,* 2000).

1.2. Global Status of Genetically Modified Organisms

Genetically modified (GM) crops have become available in the world food markets with ever increasing abundance including maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), canola (*Brassica napus* L.), squash (*Cucurbita pepo* L.), and papaya (*Carica papaya* L.) after the first marketed transgenic tomato (Flavr Savr-Calgene) was approved by regulatory authorities in 1994. Twenty-eight GM crops were approved world-wide at the end of 1997 (Hemmer,1997).

Globally, the area covered by genetically modified (GM) crops increased from 1.7 million hectares to 90 million hectares between 1996 and 2005 with a market value of $ 5.25 billion U.S. dollars (**Figure 1.1**) (James, 2005). In 2004, genetically modified (GM) crops accounted for 29 % of global crop production (James, 2004). It is estimated globally that 56 % of soybean, 28 % of cotton, 19 % of canola and 14 % of maize is genetically modified and the countries growing 99 % of GM crops are the USA, Argentina, Canada, Brazil, China, Paraguay, India and South Africa, growing 59 %, 20 %, 6 %, 6 %, 5 %, 2 %, 1 %, 1 % of global GM crops, respectively (James, 2004). Currently, two GM traits are found in commercial GM crops, namely herbicide tolerance (in 75 % of GMOs) and insect resistance (in 25 % of GMOs).

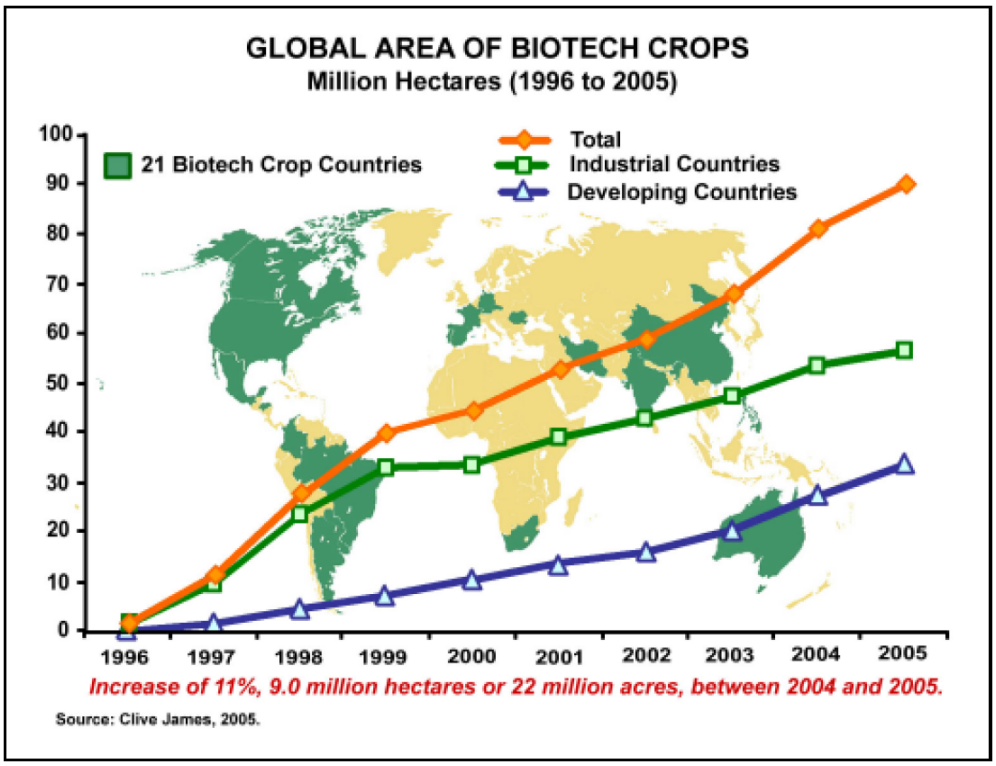

Figure 1.1. Global area of genetically modified crops between 1996 and 2005 (James, 2005).

1.3. Commercial Applications of GMOs

Transgenic plants are currently produced by introduction of genes that confer several properties such as: resistance to insects, viruses or herbicides, improved nutritional value and flavor, resistance to environmental stresses (such as drought, salinity, pollution, extreme temperatures), capacity to produce heterologous substances with pharmacological properties, prolonged organoleptic stability, extended conservation and improved value of flowers (floriculture). But not all of these traits are already present in commercially available plants or employed in edible crops. Indeed, some of them are just successful applications of recombinant DNA technology, may have an exclusive scientific value or be yet only at an experimental phase (Paparini and Romano-Spica, 2004).

Currently, the most common application area of transgenic plants is developing resistance to herbicides that are used for weed management. Weeds fight against crop plants by competing for limited sources of water, light and nutrients. In worst cases,

weeds' infestation can kill crops with a dramatic loss in yield percentage. Transgenic herbicide resistance crops that metabolize particular herbicides have been developed and commercialized. Glyphosate (Roundup®) is a non - selective herbicide used on many food and non-food field crops. It controls weeds by inhibiting the enzyme EPSP (5 - enolpyruvylshikimate - 3 - phosphate) synthase which is necessary for the growth of plants by involving in the production of aromatic amino acids biochemical pathway. GM crops contain the gene CP 4 EPSP synthase isolated from *Agrobacterium sp.* strain CP 4. The gene produces the modified form of the EPSP synthase enzyme that is tolerant to glyphosate (Deisingh and Badrie, 2005). In 1996, Monsanto company introduced the first glyphosate-resistant crop, Roundup Ready® soybeans and this was followed by cotton, corn, beet and canola (EPA, 2002). LibertyLink® crops are engineered to be resistant to glufosinate ammonium (sold as Liberty® and Basta® herbicide) by metabolizing it. Glufosinate ammonium, also called phosphinothricin (PPT), is essentially a synthetic, truncated version of a toxin produced by *Streptomyces hygroscopicus*. It inhibits the enzyme glutamine synthetase leading to the accumulation of pyhtotoxic levels of ammonia killing the plants. The gene conferring tolerance to phosphinothricin, was isolated from the soil microbe *Streptomyces viridochromogenes* and encodes the enzyme phosphinothricin-N-acetyl transferase (PAT) that inactivates glufosinate (Viljoen, 2005). In 2005, being the dominant trait, herbicide tolerance deployed in soybean, maize, canola and cotton occupied 71 % or 63.5 million hectares of the global biotech 90 million hectares (James, 2005).

Resistance to pests is the second most widely used trait in the application of transgenic crops (after herbicide resistance) in world agriculture (James, 2005). The first genes available for genetic engineering of crops for pest resistance were the *Cry* genes of naturally occuring soil-borne bacterium *Bacillus thuringiensis* (*Bt*). Several subfamilies of the *Cry* gene have been discovered, named and classified. They code for the Cry proteins or "Bt" toxins that can kill a large variety of host insects and even nematodes with a high degree of specificity (Wei *et al.,* 2003). The genes code for the crystal like 'Cry' proteins, a delta-endotoxin, during spore formation which, when ingested by susceptible insect larvae, act as poisons. The endotoxin binds to receptors in the midgut of insects distrupting ion flow, causing gut paralysis and

subsequent death. Mammals do not have these receptors and are unaffected by this endotoxin (Schnepf *et al.,* 1998). Transgenic plants, modified to express Bt toxins, also known as Bt - protected plants, were first created during the early 1990s by inserting the *Cry* genes into different crops varieties (Tian *et al.,* 1991). In 1995, the first insect resistant corn, cotton and potato were approved for market release in US. In 2005, Bt crops occupied 18 % of the global biotech areas with 16.2 million hectares. 11 % of the global biotech area (10.1 million hectares) is occupied by the transgenic crops having stacked genes for the two traits herbicide tolerance and insect resistance (James, 2005).

Another well known application of transgenic crops is enhancing the nutritional quality of plants and Golden Rice® which was created to obtain a functioning provitamin A (beta carotene) biosynthetic pathway in rice endosperm was a success story in this regard (Ye *et al.,* 2000). The transgenic plant accumulates beta carotene in the endosperm and may provide a supplementary dietary source of provitamin A for people feeding mainly on rice.

1.4. Developing a Genetically Modified Organism

A new transgenic crop or a GMO can be developed through a complex, serial procedure (**Figure 1.2**) (Paparini and Romano-Spica, 2004). For the successful transformation of plants certain criteria have to be met. The basic requirements for transformation can be listed as follows;

- Target tissues competent for propagation or regeneration.
- An efficient DNA delivery method.
- Agents to select for transgenic tissues.
- The ability to recover fertile transgenic plants at a reasonable frequency.
- A simple, efficient, reproducible, genotype-independent and cost-effective process.
- A tight time frame in culture to avoid somaclonal variation and possible sterility.

At present, three techniques namely, biolistics or microprojectile bombardment, protoplast transformation and *Agrobacterium*-mediated transformation appear to fulfill these criteria.

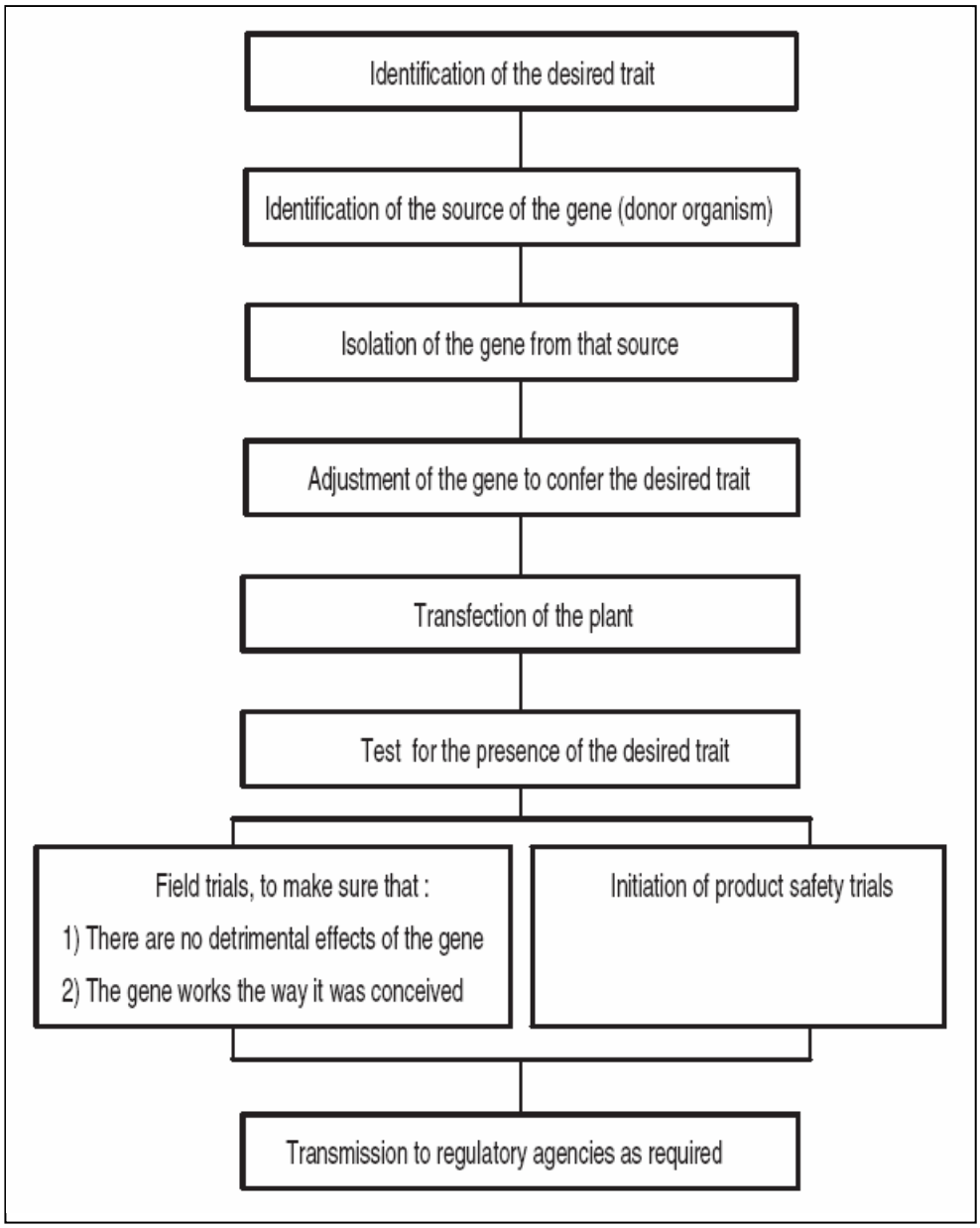

Figure 1.2. Development of a transgenic plant (Paparini and Romano-Spica, 2004).

In protoplast transformation method, protoplasts are isolated either by a mechanical or by an enzymatic process to remove the plant cell wall. This results in the production of a suspension containing millions of individual cells and therefore offers the advantage of probable single cell targets. Protoplasts are frequently obtained from an established suspension cell line of callus initiated from immature embryos, immature inflorescences, mesocotyls, immature leaf bases and anthers (Maheshwari *et al.*,1995). However, plant cell wall regeneration required after transformation and reduced yields are observed.

Biolistics is the delivery of microprojectiles, usually of tungsten or gold coated with DNA and propelled into the target cells by acceleration. The acceleration can be provided by gun powder, by gases, such as helium or CO_2, or by an electric discharge. This method can introduce DNA into virtually any tissue from any cultivar and success depends critically upon the ability of the target tissue to proliferate and give rise to a fertile plant (Hansen and Wright, 1999).

Agrobacterium genetically transforms its host by transferring a well - defined DNA segment from its tumor - inducing (Ti) plasmid to the host - cell genome (Gelvin, 1998). In nature, the transferred DNA (T-DNA) carries a set of oncogenes (Gaudin *et al.*, 1994) and opine - catabolism genes, the expression of which, in plant cells, leads to neoplastic growth of the transformed tissue and the production of opines, amino acid derivatives used almost exclusively by the bacteria as a nitrogen source. Recombinant *Agrobacterium* strains, in which the native T - DNA has been replaced with genes of interests, are the most efficient vehicles used today for the introduction of foreign genes into mostly dicotyledoneus plants and for the production of transgenic plant species (Draper *et al.,* 1988).

1.5. Structure of a "Transgene" in GMOs

GMOs typically contain an insert or gene "cassette" consisting of a "promoter" (P), that controls the expression of the transgene, "the encoding region", that defines the sequence of amino acids of a particular gene and an "expression terminator" (T) that functions as a stop signal to terminate the "reading" of the gene during protein production (**Figure 1.3**). Common promoter and terminator elements are used in combination with most transgenes to ensure adequate expression of the transgenes in the host organism. A gene construct can also contain a marker gene such as antibiotic resistance.

The promoter of the transgene which locates at the 5' end of the gene cassette, is one of the most critical choices to make since its sequence can affect the expression level of the transgene, the historical fate of the product and time of the synthesis (Paparini and Romano-Spica, 2004).

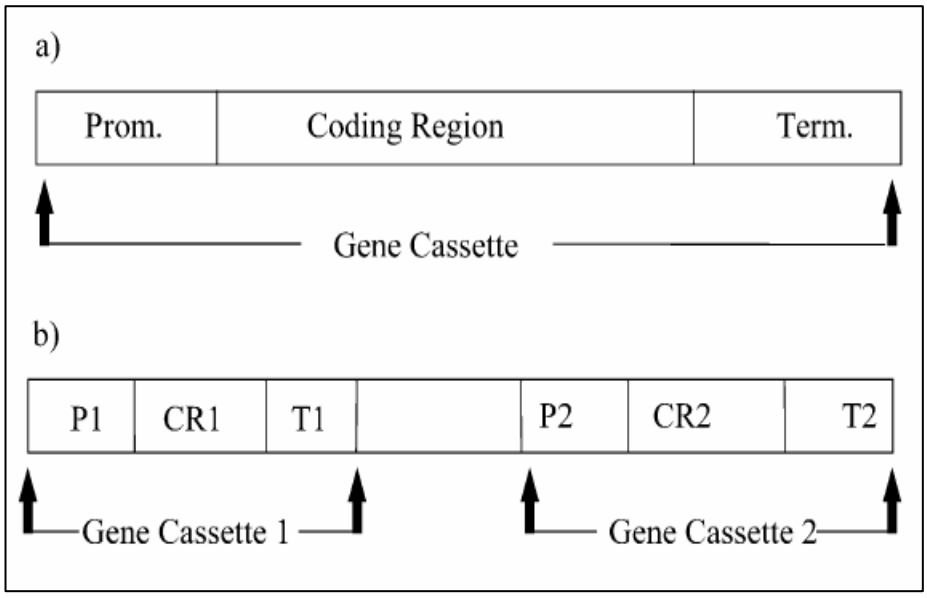

Figure 1.3. (a) Schematic representation of a gene cassette, consisting of a promoter P, a structural gene (coding region) and a terminator T; (b) two cassettes integrated into the host genome (Minunni, 2003).

Most of the transgenic crops approved and commercialized utilize the constitutive promoter of the subunit 35S of ribosomal RNA of Cauliflower Mosaic Virus (CaMV). Currently approved GM crops contain more than 39 transgenes. Among the most common are the bacterial neomycin-phosphotransferase II gene (*npt II*), the phosphinothricin acetyl transferase (PAT) gene from *S. hygroscopicus (BAR)* , the *Cry* genes from *Bacillus thuringiensis* and the 5 - enolpyruvylshikimate - 3 - phosphate synthase (EPSPS) gene. The third element of the gene cassette is the terminator which situates at the 3' end of the transgene. It functions as a regulatory sequence controlling the halt of the transcription by RNA polymerase and the poly-adenylation signal. T - NOS from *Agrobacterium tumefaciens* is widely used as a terminator sequence for the production of many transgenic vegetables commercially available.

1.6. Regulation of Genetically Modified Organisms (GMOs)

Genetically modified organisms are the fact of modern agriculture and the application of biotechnology to agricultural production has resulted in a number of whole and processed foods in the world marketplace today (Mitten *et al.,* 1999). As

the production and consumption of transgenic crops increases, possible risks to the environment and potential hazards to human health has started to be questioned and the safety of foods derived from genetically modified organisms has become the center of interest worldwide. Debates in various sectors of society raised different points of view regarding food safety, allergic reactions and transfer of antibiotic resistance genes as well as the possibility of risks to the environment caused by dissemination of transgenic crops or their foreign genes that could, for instance, transfer herbicide tolerance to weeds, develop resistant pests or kill useful insects (Azavedo and Araujo, 2003), as well as other concerns (http://rama.ceng.metu.edu.tr/gdo).

1.6.1. Safety Assessment of Genetically Modified Organisms (GMOs)

The first international and national provisions for the safety assessment and regulation of genetically modified organisms (GMOs), including GM crops and derived foods were drawn up by scientific experts in the mid-1980s (OECD, 1987; US OSTP, 1986). The Organization and Environmantal Development (OECD) and the World Health Organization (WHO) have embraced the concept of "substantial equivalence" as the cornerstone of GMO food safety assessment. Authorities and agencies involved in food safety assessment in most countries have based their safety assessment strategies and guidelines on this approach (UK Department of Health, 1991; US FDA, 1992; Health Canada, 1994; Japan MHLW, 2000; European Commission 1997b). According to this concept, the allergen, nutrient and toxin content of the new GM food must fall within the normal range of the equivalent, conventional food. The concept of substantial equivalence provides the framework for a comperative approach to identify the similarities and differences, between GMOs and their traditional counterparts that have a known history of safe use (Paparini and Romano-Spica, 2004).

Today, many countries including EU, Japan, US, Canada, Australia, Korea, have their own regulatory framework for safety assessment of foods made or derived from genetically modified organisms, however, they are not necessarily consistent in the basic approach or methodologies (Endo and Boutrif, 2002). Two types of regulatory

frameworks for foods derived from GM crops can be distinguished. Some jurisdictions enacted specific 'process-based' legislation for the regulation of all genetically modified organisms, these include the European Union (EU) and Australia. In contrast, other regulatory systems are 'product-based', focusing on the resulting product characteristics and use, and not on the process of genetic modification, as for instance those in the United States of America (USA) and Canada (König *et al.,* 2004). Attemptions to agree on common rules at an international level resulted in start of negotiations that are carried out in regular meetings of specialised Codex Committees established by FAO and WHO, e.g. the Codex Committee on Food labelling of pre-packaged foods. The commonly defined rules can then be implemented in the participating countries, on a voluntary basis. The main mission of *Codex Alimentarius* is to protect consumers health and to ensure fair practices in international food trade (Endo and Boutrif, 2002; Cheftel, 2005). Also, an agreement, the 'Cartagena Biosafety Protocol', puts into effect rules that govern the trade and transfer of GMOs across international borders (e.g. labeling of shipments of GM commodities), and allows governments to prohibit the import of GM food when there is concern over its safety (Gupta, 2000).

The basic steps of the hazard identification and characterisation of GM crops and elements of safety assessment of food derived from a GM crop (in **Figure 1.4**) are summarized in the review of König *et al.,* (2004). Hazard identification and characterisation of GM crops are conducted in four steps:

(i) Characterisation of the parent crop and any hazards associated with it;

(ii) Characterisation of the transformation process and of inserted recombinant DNA (the potential consequences of any gene transfer event of the recombinant DNA to microbes or humans should also be assessed);

(iii) Characterisation of the introduced proteins (their potential toxicity and allergenicity) and metabolites; and

(iv) Identification of any other targeted and unexpected alterations in the GM crop, including changes in the plant metabolism resulting in compositional changes and assessment of their toxicological, allergenic, or nutritional impact.

Any identified differences are then further assessed as to whether they might have adverse implications for human health in the range of exposure scenarios. The concept of substantial equivalence is thus the starting point and guiding concept for the safety assessment, not its conclusion (FAO/WHO, 2000; Codex Alimentarius Commission, 2003b, König *et al.,* 2004).

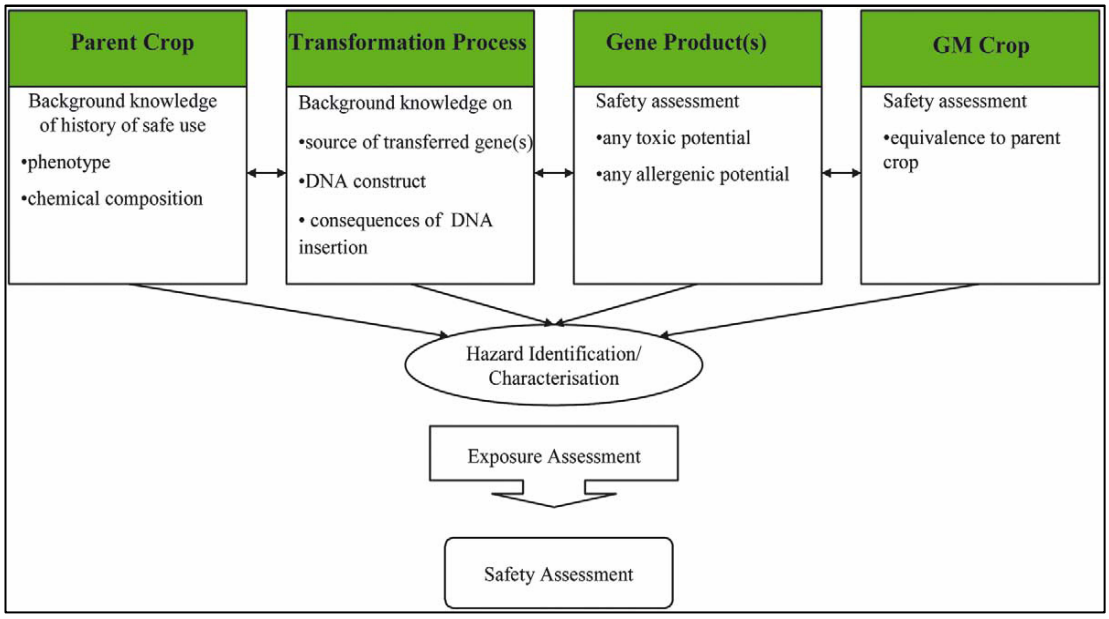

Figure 1.4. The elements in safety assessment of food derived from a GM crop (König *et al.,* 2004)

Main points and comments related to protection of nature and biodiversity regarding to GMOs in EU Directives and Regulations are summarized in recent article of Arvanitoyannis *et al.,* 2006.

1.6.2. Labelling and Traceability of Genetically Modified (GM) Crops

Traceability of genetically modified crops plays a role in increasing public confidence in biotechnology since it forms an essential component of any risk management strategy and is a key requirement for post - marketing surveillance. Legal requirements for provisions to trace back food ingredients derived from GM crops through the agrofood chain have two main objectives: enabling withdrawal of foods or feed derived from GM crops in case of the appearance of unforeseen

adverse effects, and providing consumers choice between foods containing - or free of - GM crops through labelling (Kuiper *et al.,* 2004).

Labelling regulations for transgenic crops were first introduced in Europe. Labelling of foods derived from GM crops containing detectable transgenic material is required by EU legislation since 1998 (EU Regulations 1139/98 and 49/2000) (Kuiper *et al.,* 2004). The European Union (EU) is currently enacting more stringent policies requiring traceability for all GM crops and mandatory labels on all food and feed products if the proportion of GM ingredient exceeds a threshold level of 0.9% defined in the EU Labelling and Traceability Regulation (Regulation 1830/2003).

In the report of 2006 Biotechnology Policy of European Comission, traceability and labelling requirements for GM products set out in the EU Regulation 1830/2003 are summarized (EC, 2006). The Regulation covers all GMOs that have received EU authorisation for their placing on the market, namely all products containing or consisting of GMOs, including food and feed. Examples include GM seeds and bulk quantities or shipments of whole GM grain, such as soybean and maize and as well as the food and feed derived from a GMO such as flour produced from genetically modified maize (EC, 2006).

For all pre - packaged products consisting of or containing authorised - GMOs exceeding 0.9 %, the Regulation 1830/2003 requires that operators indicate on a label: “This product contains genetically modified organisms” or “This product contains genetically modified [(name of organism(s))]”. For non pre - packaged products offered to the final consumer or to mass caterers, these words must appear on, or in connection with, the display of the product (EC, 2006).

In addition, the Regulation (EC) 1829/2003 defines specific conditions that allows up to a maximum of 0.5 % presence of non-authorised GMOs in a feed or food which have received a positive assessment in terms of safety for health and the environment but above 0.5 %, it is prohibited to put the product on the market (EC, 2006).

The regulatory issues of risk analysis and labelling are currently harmonised by *Codex Alimentarius*. The implementation and maintanance of the regulations necessitates sampling protocols and analytical methodologies that allow for accurate determination of the content of genetically modified organisms within a food and feed sample (Miraglia *et al.,* 2004).

1.7. Detection of Genetically Modified Organisms (GMOs)

Legislation enacted worldwide to regulate the presence of genetically modified organisms (GMOs) in crops, foods and ingredients, necessitated the development of reliable and sensitive methods for GMO detection (Ahmed, 2002).

Post-commercialization tracking of GM crops requires three types of analytical tests; (i) detection (Screening of GMOs), (ii) identification and (iii) quantification.

(i) Detection; The objective is to determine if a product contains a GMO or not. For this purpose, a screening method can be used. The result is a positive/negative statement. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results in all control laboratories, which can be achieved through inter-laboratory validation.

(ii) Identification; The purpose of identification is to reveal how many different GMOs are present and if they are authorized or not. Specific information (i.e. details on the molecular make-up of the GMOs) has to be available for the identification of GMOs.

(iii) Quantification; If a food product has been shown to contain (one or more) authorised GMOs, then it become necessary to assess compliance with the threshold regulation by the determination of the amount of each of the GMOs present in the individual ingredients from which it has been prepared (Bonfini *et al.,* 2002).

The detection can be accomplished by qualitative methods (presence or absence of promoters, terminators or transgenes), whereas the third stage uses semiquantitative

(above or below a threshold level) or quantitative (weight/weight % or genome/genome ratio) methods. Currently, the two most important approaches are immunological assays using antibodies that bind to the novel proteins and PCR-based methods using primers that recognize DNA sequences unique to the GM crop (Auer, 2003).

1.7.1. Protein-based Methods

GM products contain an additional trait encoded by an introduced gene, which produce an additional protein that confers the trait of interest. Certain proteins may be expressed only in specific parts of the plant (tissue - specific promoters are already being used for specific purposes) or expressed at different levels in distinct parts or during different phases of the physiological development (Bonfini *et al.,* 2001). The expression levels of transgene products in plants were reported to be in the range of 0 - 2 % of the total soluble protein, even when strong constitutive promoters were used to drive expression (Longstaff *et al.,* 1995).

Protein detection methods are based mainly on immunoassays. Immunoassay technologies with antibodies are ideal for qualitative and quantitative detection of many types of proteins in complex matrices when the target analyte is known (Brett, 1999). Both monoclonal (highly specific) and polyclonal (often more sensitive) antibodies can be used depending on the amounts needed and the specificity of the detection system (e.g. antibodies to whole protein or specific peptide sequences), depending on the particular application, time allotted for testing and cost. Immunoassays with antibodies attached to a solid phase have been used in two formats: a competitive assay in which the detector and analyte compete to bind with capture antibodies, or a two - site (double antibody sandwich) assay in which the analyte is sandwiched between the capture antibody and the detector antibody (Ahmed, 2002). Illustration of the principles of the immunological test is given in **Figure 1.5 (a)**. The most common immunological assays are Western blot, enzyme - linked immunosorbent assays (ELISA) and immunochromatographic assays (lateral flow strip tests).

1.7.1.1. Western Blot

The western blot is a highly specific method that provides qualitative results suitable for determining whether a sample contains the target protein below or above a predetermined threshold level. The samples to be assayed are solubilized with detergents and reducing agents, and separated by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis. These components are transferred to a solid support (usually a nitrocellulose membrane), and binding immunoglobulin sites on the membrane are blocked by dried nonfat milk. The specific sites are then probed with antibodies (either a high-titer polyclonal antiserum or a mixture of monoclonal antibodies raised against the denatured antigenic epitopes). Finally, the bounded antibody is stained with Ponceau, silver nitrate or Coomassie, or a secondary immunological reagent, such as protein A coupled to horseradish - peroxidase (HRP) or alkaline phosphatase (Sambrook and Russel, 2000). The detection limits of western blots vary between 0.25 % for seeds and 1% for toasted meal (Yates, 1999). This method, however, is considered more suited to research applications than to routine testing.

1.7.1.2. Enzyme-Linked Immunosorbant Assay (ELISA)

The ELISA tests are also based on antibodies recognising their target protein. They can be in microwell plate (microtiter) or strip (lateral flow strip assay) format. The antibody - coated microwells, with removable strips of 8 - 12 wells, are quantitative, highly sensitive, economical, provide high throughput and are ideal for quantitative high - volume laboratory analysis, provided the protein is not denatured (Ahmed, 2002). ELISA detection limits for CP4 EPSPS soybean protein was 0.25 % for seeds and 1.4 % for toasted meal (Yates, 1999). The lateral flow strip assay is a variation on ELISA using strips rather than microtiter wells. It is a qualitative test and can be used in the field.

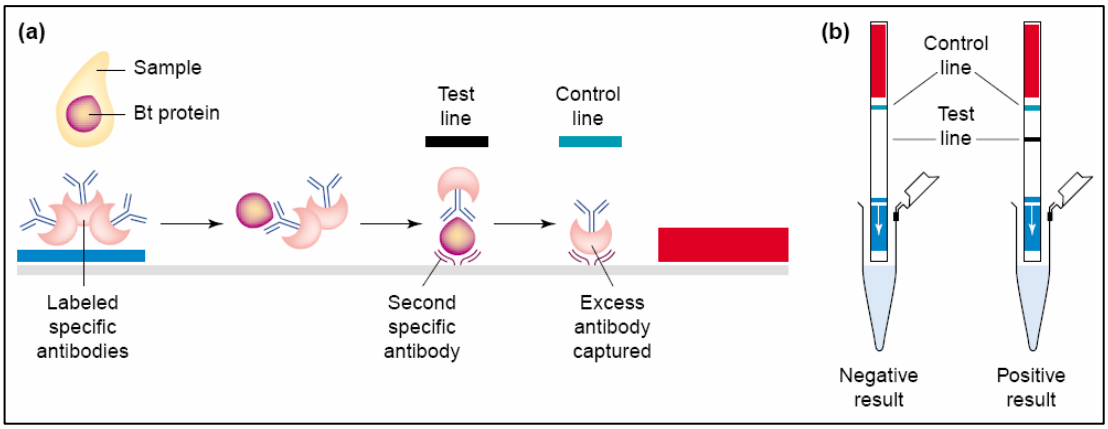

Figure 1.5. Lateral flow strip assay format (original by D. Layton). **(a)** Side view illustrating principles of the immunological test, and relative location of control and test lines on a nitrocellulose strip. **(b)** Vertical view of test strips dipped in an eppendorf containing genetically modified material, and showing both negative and positive test results. Abbreviation: Bt, *Bacillus thuringiensis*. (source; Ahmed, 2002).

Immobilized double antibodies, specific for the expressed protein, are coupled to a color reactant and incorpotrated into a nitrocellulose strip, which, when placed in an eppendorf vial containing an extract from plant tissue harboring a transgenic protein, leads to an antibody sandwich with some of the antibody that is coupled to the color reagent. This colored sandwich flows to the other end of the strip through a porous membrane that contains two captured zones, one specific for the transgenic protein sandwich and another specific for untreated antibodies coupled to the color reagent. The presence of only one (control) line on the membrane indicates a negative sample, and the presence of two lines indicates a positive result (Ahmed, 2002). Illustration of test strips dipped in a sample containing genetically modified material is shown in **Figure 1.5 (b)**.

However, ELISA tests are prone to cross-reactivity giving false-positive results for similar protein structures. Therefore these test systems need to be throughly validated and specified according to the scope. ELISA for the specific detection of the protein CP4-EPSPS in Roundup Ready® soybean has been tested and validated in an interlaboratory study organised by the European Comission Joint Research Center (Lipp and Anklam, 2000). Results indicated that the method is able to detect the presence of GMOs in raw soybean material at concentrations ranging between 0.3 % and 5 % (Bonfini *et al.*, 2001). Other microtiter ELISA systems (MON810, Starlink)

were evaluated by the American Association of Cereal Chemists (AACC). Currently, there are a range of validated ELISA systems available which are predomianantly used on unprocessed materials early in the food chain as an initial screening method at the point - of sale where time is the critical factor (Popping, 2003).

1.7.2. DNA-based Methods

DNA is the preferred analyte for almost any kind of sample (raw materials, ingredients, processed foods) as it is a rather stable molecule than proteins. DNA detection methods for GM foods rely on the complementarity of two strands of DNA double helix that hybridize in a sequence-specific manner. The DNA that has been engineered into a crop consists of several sequenced elements that govern its functioning. They are typically a promoter sequence, structural gene and a stop sequence for the gene. At present, the most commonly used DNA-based methods involve Southern blot and amplification of a specific DNA with the PCR technique.

1.7.2.1. Southern Blot

The method involves fixing isolated sample DNA onto nitrocellulose or nylon membranes, probing with double-stranded (ds) - labeled nucleic acid probes specific to the GMO, and detecting hybridisation radiographically, fluoremetrically or by chemiluminescence. Earlier probes were labeled with ^{32}P. However, nonradioactive fluorescein, digoxigenin-, or biotin-labeled DNA probes with sensitivity equal to ^{32}P probes, were recently used, eliminating the need for radioactivity in the testing laboratory (Ahmed, 2002). Recently, an alternative Southern blot technology has been attempted with near infrared (NIR) fluorescent dyes (emitting at ~700 and 800 nm) coupled to a carbodiimide-reactive group and attached directly to DNA in a 5 min reaction. The signals for both dyes are detected simultaneously (limit in the low zeptomolar range) by two detectors of an infrared imager, something not yet possible with conventional radioactive or chemiluminescent detection techniques (Stull, 2001).

1.7.2.2. PCR

The polymerase chain reaction (PCR) technology is an exploitation of DNA replication, the mechanism responsible for DNA copying in all living cells. In a standard PCR, two pairs of primers are used: (1) forward, sense or 5′→3′, and (2) reverse, antisense or 3′→5′. These primers are designed to hybridize on opposite strands of the sequence of interest, and through a series of repetitive cycles, of 2 - 3 thermal steps, amplify the sequence between the primers exponentially within reasonable time (usually less than 3 h to obtain 109 copies). Although mostly used method, the major limitations for PCR-based detection of DNA derived from genetically modified organisms (GMOs) are access to information about applicable PCR primers and access to DNA suitable for reliable analysis (Holst-Jensen *et al.,* 2003).

PCR-based GMO tests can be categorised into four levels of specificity. Each category corresponds to the composition of the DNA fragment that is amplified in the PCR (**Figure 1.6**). The least specific methods are commonly called “screening methods” and relate to target DNA elements, such as promoters and terminators that are present in many different GMOs. The second level is “gene-specific methods”. These methods normally target a part of the DNA harbouring the active gene associated with the specific genetic modification. Examples are the *Bt* gene coding for a toxin acting against certain insects or the *EPSPS* gene coding for tolerance against a specific herbicide. Both screening and gene-specific methods are based on detection of more or less naturally occuring DNA sequences, a fact that significantly increases the risk of obtaining false positive analytical results in tests. The third level of specificity is “construct-specific methods”, which target the junction between two DNA elements, such as the promoter and the functional gene. These methods target DNA sequence junctions not naturally present in nature. However, different GMOs may share several DNA elements, for example both the same promoter and gene, and sometimes even the same plasmid has been used to transform plants (e.g. the two distinct maize GMOs Mon809 and Mon810; Agbios, 2003). The highest specificity is seen when the target is the unique junction found at the integration locus between the inserted DNA and the recipient genome. These are called “event-specific

methods". Although specific enough, even the event-specific methods have their limitations. Crossbreeding between two GMO lines may lead to so called "stacked genes". For example, a herbicide-tolerant GMO can be combined with an insecticide-tolerant GMO. Both sets of functional genes are present in the crossbreed but not necessarily linked, i.e. they are likely to be situated on different chromosomes. Quantitative methods cannot distinguish between the gene-stacked GMO and a mixture of its two parental GMOs. This problem is only alleviated if the test is performed on material from a single organism, such as a leaf or a single kernel of grain or seed, in which case the presence of both target sequences is demonstrated from a single individual that consequently must be a gene-stacked breed.

In the US, this type of hybrid GMOs are not regulated if both parent GMOs are authorised. In the European Union, however, gene-stacked crossbreeds require separate authorisation and consequently require quantitation as a single GMO (Miraglia *et al.,* 2004). Examples of published methods to detect GMO derivatives grouped according to categories of specificity are rewieved in the article of Miraglia *et al.,* 2004.

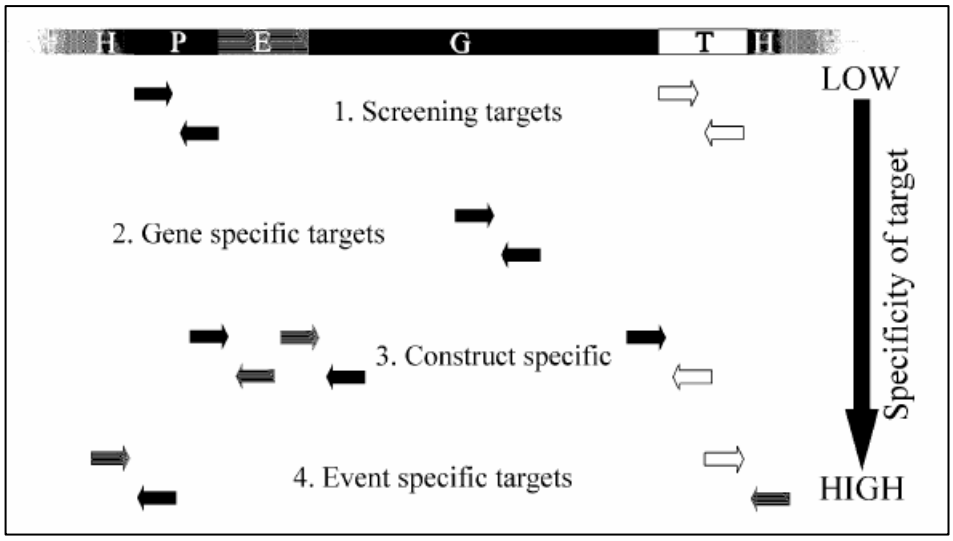

Figure 1.6. A schematic representation of a typical gene construct and four types of PCR-based assays showing increasing specificity (from *top* to *bottom*). H: host genomic DNA, P: promoter element, E: enhancer element, G: gene of interest, T: terminator. The gene construct is composed of P–T and has been inserted into H (Miraglia *et al.,* 2004).

1.7.2.2.1. PCR-based Quantitation

A crucial aspect of analysis of GMOs in food is quantitation, because maximum limits of GMOs in foods are the basis for labeling in the EU. Therefore, more quantitative PCR approaches were needed.

The first quantitative PCR tests were based on "Competitive PCR". Quantitation is done by comparison of the amount of end product (end - point quantitation), that is when the PCR reaction is completed. Two target sequences with very similar features and amplifiability are co - amplified in a single reaction tube. Because the two targets compete for available nucleotides, primers and DNA polymerases, the relative quantity of end product is assumed to correspond to the relative quantity at the beginning of the first PCR cycle. Competitive PCR requires development of suitable competitor molecules, and is highly sensitive to the starting concentrations and dilution of template DNAs. It involves massive pipetting of amplified DNA and visualisation by agarose gel electrophoresis, associated with a significant risk of cross contamination (Holst-Jensen *et al.,* 2003).

"Real-time Quantitative PCR" was introduced in order to circumvent some of the problems of conventional quantitative end-point PCR. In theory, production of PCR products should proceed exponentially. However, in practice it reaches a plateau between 30 and 40 cycles because certain reaction components become limiting. In conventional PCR, products of the reaction are measured at a single point in the reaction profile. Plotting the concentration of products present at this point as a function of the initial amount of DNA present in each of those reactions shows that proportionality between DNA concentration (dynamic range) and PCR products occurs over a limited range of DNA concentrations (**Figure 1.7**), leading to loss of precision in quantitation. However, it has been shown empirically that the concentration of DNA in real - time PCR reaction is proportional to PCR cycle number during the exponential phase of PCR. Therefore, if the number of cycles it takes for a sample to reach the same point in its exponential growth curve is known (**Figure 1.7 (c)**), its precise initial DNA (then GMO) content can be determined (**Figure 1.7 (d)**) (Ahmed, 2002).

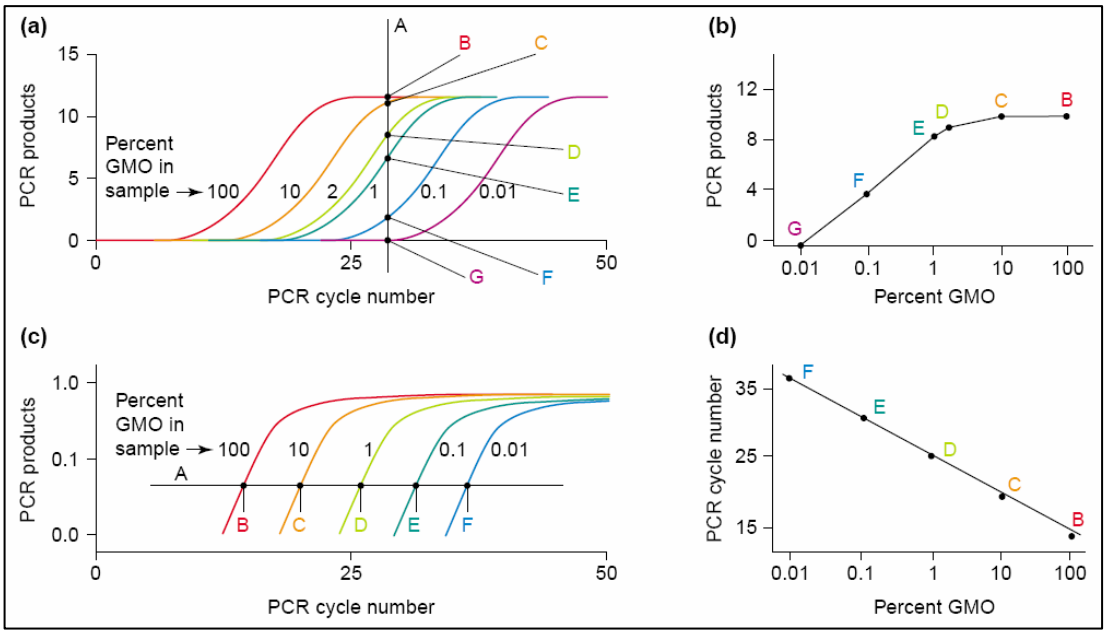

Figure 1.7. Differences between traditional PCR (a,b) and real-time PCR (c,d) (original by J. Fagan). The concentration of products present at point A (a,c) are plotted as a function of percent GMO present in the initial sample, demonstrating that proportionality between DNA concentration and PCR products (dynamic range) is limited for conventional PCR (b). By contrast, there is a linear relationship between PCR products and DNA concentration during exponential phase of real-time PCR (Ahmed, 2002).

Alternatives to competitive PCR, a method have developed where short oligonucleotide probes are labelled immediately after the PCR reaction, and where there is a linear relationship between the quantity of labelled probe and the quantity of the corresponding amplification product. The reactions are successively analysed by array hybridisation of the probes, and the identity of positives and quantitation is done by image analysis (Holst-Jensen *et al.*, 2003).

Presently, real-time PCR can be considered as the most powerful tool for the detection and quantification of GMOs in a wide variety of agricultural and food products. While multiplex PCR formats with an endogenous reference gene will be able to increase the accuracy, precision and throughput of this technique, a more comprehensive evaluation of real-time protocols and formats needs to be performed.

Besides ELISA and PCR - based detection systems, there is a significant development in direct detection of a DNA sequence via biosensors and microarrays. Both are rapid, sensitive, specific and yield results in real time. However having

each has its own problems that need to be investigated further. Biosensors are difficult to commercialize due to inherent instability issues while microarrays yield a vast amount of data which are complex for analysis (Deisingh and Badrie, 2005).

1.8. DNA-based Biosensors for Detection of GMOs

1.8.1. Biosensors

A biosensor is an analytical device that combines the specificity of a biorecognition molecule with a transducer to produce a signal proportional to target analyte concentration (Junhui *et al.,* 1997). The biorecognition molecule, such as an enzyme, antibody, sequence of DNA, peptide or even a microorganism, provides the biosensor with its selectivity for the target analyte so that the molecule of interest can be picked out by the biosensor from a matrix of many other molecules. The signal transducer determines the extent of the biorecognition event and converts it into a measurable response such as current, potential, temperature change, absorption of light, or mass increase through electrochemical, thermal, optical or piezoelectric means. Common transducers include amperometric electrodes, optical waveguides or mass sensitive piezoelectric crystals (Gooding, 2006). A shematic representation of the main components of a biosensor is illustrated in **Figure 1.8**.

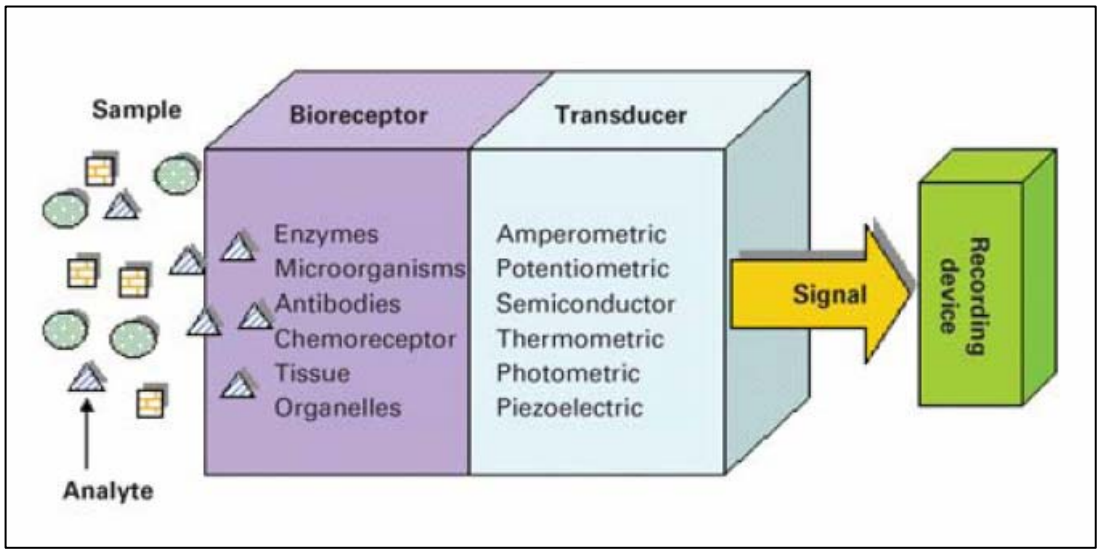

Figure 1.8. Shematic of a biosensor showing the main components (DTI, 2005)

Biosensors can be divided into two subgroups based on the biorecognition molecule. One of them is the catalytic biosensors that employ enzymes and microorganisms as biorecognition molecule which catalyses a reaction involving the analyte to give a product. Glucose is a common analyte for catalytic biosensors. The other group is the affinity biosensors. Biorecognition molecules commonly used in affinity biosensors include antibodies, receptors, nucleic acids, peptides and lectins. Affinity biosensors are characterised by a binding event between the biorecognition molecule and the analyte (the affinity reaction) with a high differential selectivity often with no further reaction occurring in a non-destructive mode. Hence the challenge is transducing the biorecognition event (Gooding, 2006).

Biosensors have been used for clinical (Wang, 2006), environmental and food control purposes (Baeumner, 2003). Recently, biosensing is the most attractive candidate for screening of genetically modified organisms in food samples (Meriç *et al.,* 2004).

1.8.2. DNA-based Biosensors (Genosensors)

Deoxyribonucleic acid (DNA) is arguably the most important of all biomolecules. The unique complementary structure of DNA between the basepairs adenine/thymine and guanine/cytosine has been the basis for genetic analysis. The ability of a single stranded DNA (ssDNA) molecule to hybridise to its complementary strand in a sample is the foundation of DNA-based detection systems (Junhui *et al.,* 1997). Hybridisation methods used today, such as microtitre plates or gel-based methods, are usually quite slow, requiring hours to days to produce reliable results, thus biosensors offer a promising alternative for much faster hybridisation assays.

DNA-based biosensors are often based on the immobilisation of a fragment of DNA with a specific sequence (the probe) on the solid support (the transducer) and on the monitoring and recording the variation of the transducer signal when the complementary fragment (the target) in solution interacts with the probe forming a stable complex (Minunni, 2003). Biosensors presently being developed for the

detection of DNA hybridisation are mostly based on electrochemical, optical and surface acoustic wave or piezoelectric transducers.

1.8.2.1. DNA Biosensors based on Electrochemical Transducers

Electrochemical detection of hybridisation is mainly based on the differences in the electrochemical behaviour of the labels with or without double - stranded DNA (dsDNA) or single - stranded DNA (ssDNA). The labels for hybridisation detection can be anticancer agents, organic dyes, metal complexes, enzymes or metal nanoparticles. There are basically four different pathways for electrochemical detection of DNA hybridisation: (1) A decrease/increase in the oxidation/reduction peak current of the label, which selectively binds with dsDNA / ssDNA, is monitored. (2) A decrease/increase in the oxidation / reduction peak current of electroactive DNA bases such as guanine or adenine is monitored. (3) The electrochemical signal of the substrate after hybridisation with an enzyme-tagged probe is monitored. (4) The electrochemical signal of a metal nanoparticle probe attached after hybridisation with the target is monitored (Kerman *et al.,* 2004).

Most strategies require an external redox - active tag or mediator except the detection of oxidation signals from the guanine and adenine bases. The electrochemical signals obtained from free adenine and guanine bases decrease on binding to their complementary thymine and cytosine bases after hybridisation. Oxidation of guanine and adenine at solid electrodes requires high - applied potentials and the associated high background currents must be subtracted to reach competitive detection limits. The use of inosine - substituted probes in which the inosine moiety prefentially forms base pair with the target cytosine residue works for this purpose with its oxidation signal well separated from the guanine response (Wang *et al.,* 1998). The inosine (I) substituted probe shows no electrochemical signal, since inosine is electro - inactive. Direct and convenient detection of the DNA hybridisation can thus be accomplished through the appearance of the target guanine oxidation signal without use of a label or redox molecules in a short time. Label - free electrochemical detection of DNA hybridisation is illustrated in **Figure 1.9** (Kerman *et al.,* 2004).

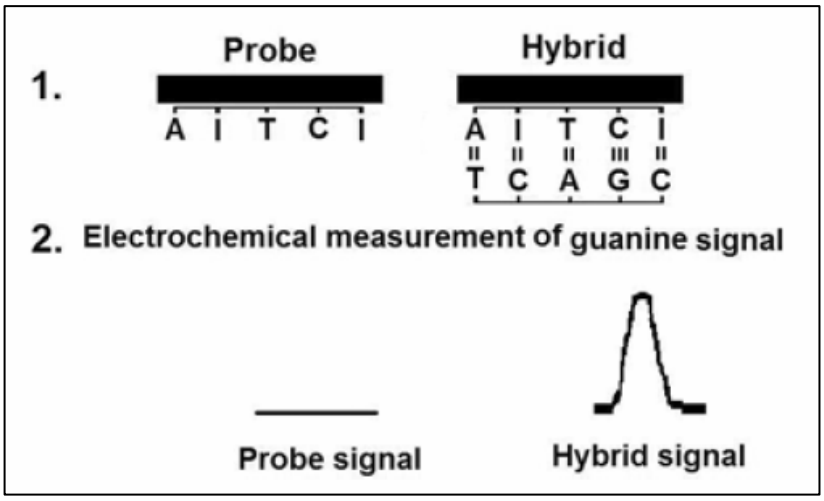

Figure 1.9. Label - free electrochemical detection of DNA hybridisation eliminates the use of redox molecules, and shortens the assay time. The inosine (I) substituted probe shows no electrochemical signals, since inosine is electro-inactive. After hybridisation with the target DNA (1), the appearance of the guanine (G) oxidation signal provides specific detection (2). (Kerman *et al.*, 2004)

1.8.2.2. DNA Biosensors based on Optical Transducers

Contemporary methods for optical sensing of chemical and biological samples at present are based mainly on interferometry, surface plasmon resonance (SPR), and luminescence. The recent technique of luminescence quenching using molecular beacons is an alternative. The hybridisation of DNA and RNA targets to probes that are bound to planar surfaces can be recognized using surface plasmon resonance (SPR). Illimunated with polarised light, the refractive index of array changes when DNA molecules hybridise to the complementary sequence on the sensor surface (**Figure 1.10**). The refractive index is directly proportional to the mass change on the chip surface and are shown in a 'sensorgram' as resonance units (RU) plotted against time (**Figure 1.11**). From the results, quantitative information on stoichiometric and kinetic data (association and dissociation rate constants, *ka* and *kd*) and affinity constants (K_D) can be determined. It allows characterisation of molecular interactions involving proteins, small molecules, carbohydrates, lipids, nucleic acids, or even whole cells (bacteria, viruses). This technology requires no labelling of the molecules and enables a real - time monitoring of the hybridisation process during an experiment (Brandt and Hoheisel, 2004).

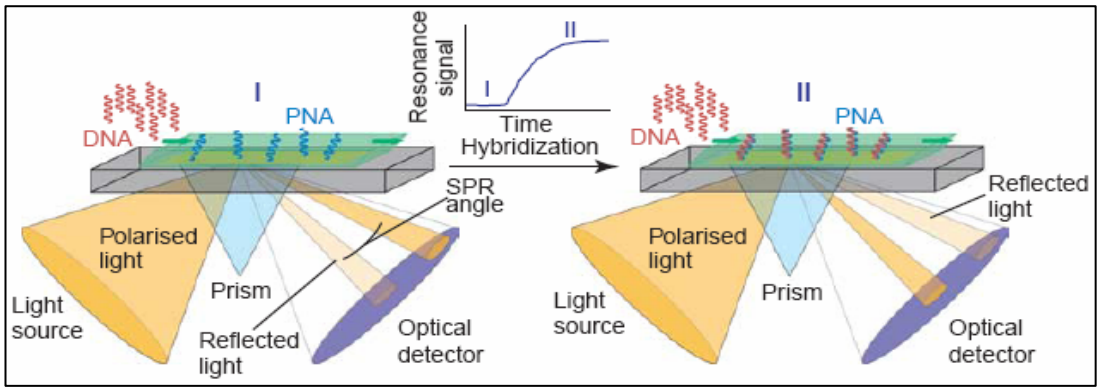

Figure 1.10. Surface plasmon resonance biosensors. Polarized light is reflected from a conductive film at the interface between the chip and the fluid containing the sample. Upon hybridisation of the peptide nucleic acids (PNAs) with DNA molecules, the refractive index changes, resulting in a resonance signal that can be measured and plotted on a sensorgram (Brandt and Hoheisel, 2004).

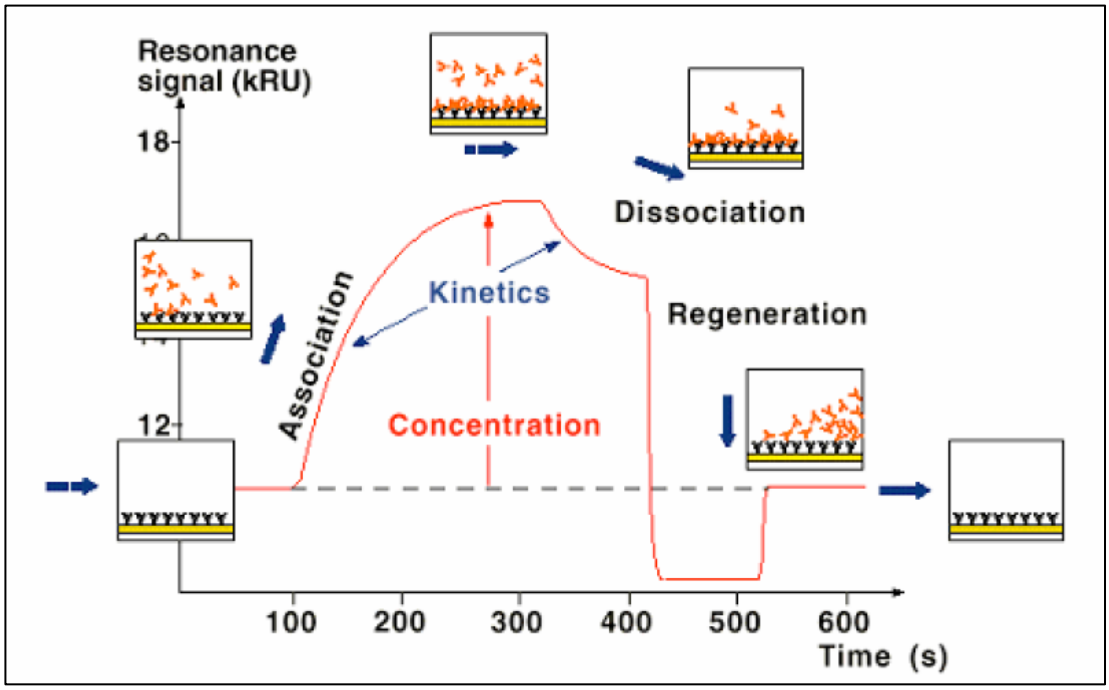

Figure 1.11. A sensorgram (source; http://www.biacore.com)

In luminescence the change in intensity or lifetime of fluorescent probe molecules called luminophores or fluorophores are sensed. Recently, molecular beacons are introduced as a new strategy in luminescence based detection systems (Tyagi and Kramer, 1996).

Molecular beacons have a fluorescent dye at one terminus and a quencher molecule at the other. Without a hybridisation partner, the conformation of a beacon is such that fluorophore and quencher lie next to each other and the molecule does not emit a signal. Upon hybridisation, the molecule stretches out, separating the quencher and the fluorophore. Without the quenching effect, the fluorescence dye emits a signal, thus reporting the occurrence of hybridisation (Brandt and Hoheisel, 2004.)

Interferometry systems are based on production of interference. Light from a source is split up by beam splitters enabling one light path to pass through a sample. The other light path is used as a reference. After passing through the sample, the two light paths are brought together to interfere and produce a set of interference fringes. Each interference fringe represents differences in phase between the sample and reference light paths. This phase, and thus the position of the fringe, give information about the sample (Ince and Narayanaswamy, 2006).

1.8.2.3. DNA Biosensors based on Piezoelectric Transducers

DNA-acoustic wave biosensors have been employed to study the duplex formation at the sensor surface and for monitoring a wide variety of processes involving nucleic acid chemistry at the solid - liquid interface without the need of radiochemical or fluorescence labels. Piezoelectric phenomena are related to the reversible electric polarisation generated by mechanical strain in crystals that do not display a centre of symmetry. The signal produced by acoustic wave devices is generated by bulk or surface acoustic waves launched by metal transducers at ultrasonic frequencies. Such waves are propagated through piezoelectric materials, usually quartz, where properties such as the orientation and thickness of the crystal, as well as the geometry of the transducer, determine the characteristics of the wave motion (Brett, 2005).

1.8.2.3.1. Piezoelectric Quartz Crystal Microbalances

The term ‘‘piezoelectric’’ derived from the Greek word piezen meaning ‘‘to press.’’ The first investigation on the piezoelectricity was performed in 1880 by Jacques and Pierre Curie who observed that a mechanical stress applied to the surfaces of various

crystals, caused a corresponding electrical potential across the crystal, whose magnitude was proportional to the applied stress. The Curies also verified the converse piezoelectric effect in which application of a voltage across these crystals caused a corresponding mechanical strain. Application of an alternating electric field across the crystal substrate results in an alternating strain field. This causes a vibrational, or oscillatory, motion in the crystal, resulting in the generation of acoustic standing waves. Depending on various criteria, the oscillator exhibits a strong preference to vibrate at a characteristic resonant frequency. The most used devices in biosensors are generally bulk acoustic wave (BAW) - based employing AT - cut quartz crystals.

The quartz crystal microbalance (QCM) is an extremely sensitive mass sensor, capable of measuring mass changes in the nanogram range. Simply, QCM is microbalance that is capable of measuring the weight of a single molecule. The heart of the QCM is the piezoelectric AT - cut quartz crystal sandwiched between a pair of electrodes (Au, Ag or Pt) (**Figure 1.12**).

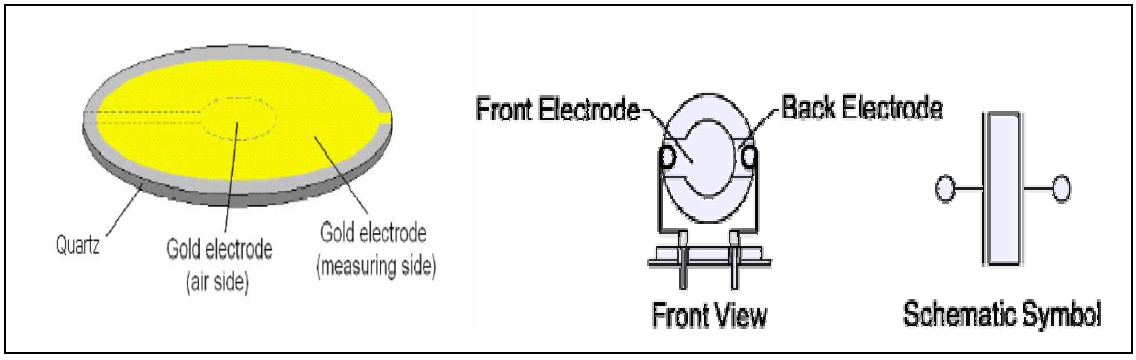

Figure 1.12. Shematic representation of a typical piezoelectric crystal.

An AT - cut crystal is typically cut at an angle of +35°15' and has a zero frequency temperature coefficient at or near room temperature that results in minimal frequency changes due to temperature (Janshoff and Steinem, 2001). AT - cut crystals oscillate in the thickness shear mode (Bruckenstein and Shay, 1985).

The first quantitative investigation of the piezoelectric effect was performed by Sauerbrey who derived the relationship for the change in frequency ΔF (in Hz) caused by the added mass Δm (in g) **(I)**:

$$\Delta F = -\frac{2F_0^2}{A\sqrt{\mu_Q \rho_Q}} \cdot \Delta m \qquad \textbf{(I)}$$

where F_0 is the fundamental resonant frequency of unloaded quartz, μ_Q is the shear modulus of AT - cut quartz ($2.947 \cdot 1011$ g.cm $^{-1}s^{-2}$), ρ_Q is the density of the quartz (2.648 g.cm^{-3}) and A is the surface area in cm^2 (Sauerbrey, 1959; Thomson *et al,* 1991; Chang *et al*, 2000). The Sauerbrey equation assumes a uniform distribution of mass on the entire electrode portion of an AT - cut quartz crystal ($\Delta f = - C_f \cdot \Delta m$).

When the electrodes are connected to an oscillator and an AC voltage is applied over the electrodes the quartz crystal starts to oscillate at its resonance frequency due to the piezoelectric effect (**Figure 1.13**).

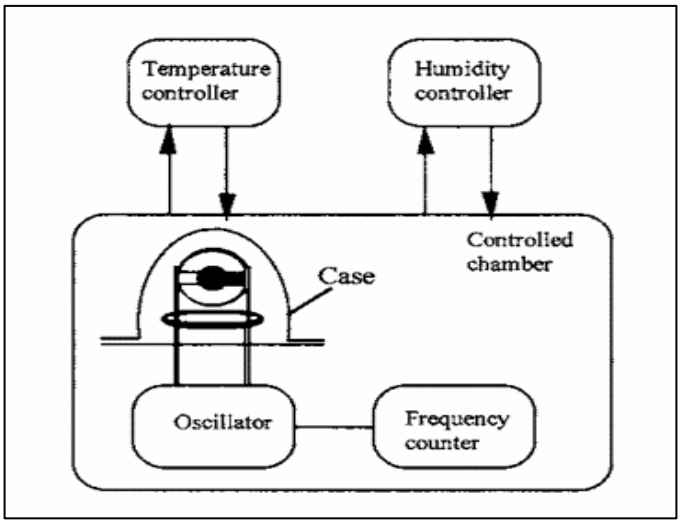

Figure 1.13. Shematic drawing of a QCM system (Mutlu *et al.,* 2002).

1.8.2.3.1.1. Principle of Piezoelectric Measurement

Frequency change versus time for the hybridisation of complementary DNA oligonucleotide with biotin - ssDNA oligonucleotide immobilised on streptavidin-coated QCM electrode (**Figure 1.14**). After hybridisation reaction the surface has been regenerated.

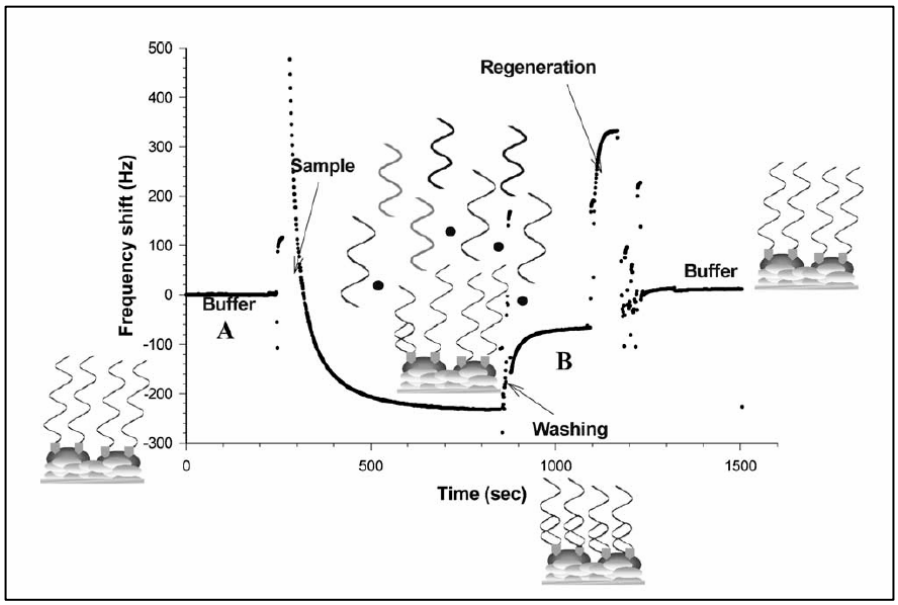

Figure 1.14. Hybridisation of complementary DNA oligonucleotide with biotin-ssDNA oligonucleotide immobilised on streptavidin-coated QCM surface (Mannelli *et al.,* 2003).

1.8.2.3.2. Probe Immobilisation Techniques to Sensor Surface

Probe immobilisation is a fundamental step in DNA-based piezoelectric biosensor development. Often, the detection limits and, in general, the analytical performances of the biosensor can be improved by optimising the immobilisation procedure on the quartz surface (Tombelli *et al.,* 2002; Zhou *et al.,* 2001). Actually, the limitation of QCM devices is nonspecific adsorption of molecules present in real matrices, since QCM is a mass sensor and any molecule able to bind or to be adsorbed on the surface is a potential interference. Moreover, receptors, in this case DNA, must be attached to the solid support retaining native conformation and binding activity.

This attachment must be stable over the course of a binding assay and, in addition, sufficient binding sites must be presented to the solution phase to interact with the analyte. Many coupling strategies utilize a chemical linker layer between the sensor base (e.g., the gold layer) and the biological component to achieve these ends. Functionalised alkane thiols form stable self-assembled layers on planar surfaces (Ulman, 1996) and act as ideal linkers (Cooper, 2003). Studies reported that crosslinker procedures using thiols or the interaction between avidin and biotinylated molecules provided a long sensor lifetime and an increased stability against degradation during the regeneration process (Uttenthaler, 1998).

Two different procedures are reported, both starting from a gold surface. The gold surface was vaporized on both sides of AT-cut quartz crystals. The two different procedures differ in the time required for surface preparation and for the functionalisation of the nucleic acid probes. The probe, actually, to be immobilised needs first to be functionalised. The kind of chemical modifications mainly used are the addition of a biotin or a thiol group at the 5' end of the DNA strand. In this way, the probe is able to bind avidin previously immobilised on the sensor surface or directly the gold layer via the sulphur group, respectively.

1.8.2.3.2.1. Immobilisation of Thiolated Probes on Gold-Surfaces

Chemisorption of DNA probes onto transducer surfaces based on the formation of gold-thiol bonds, has been reported by some authors (Yang *et al.,* 1998; Steel *et al.,* 2000; Huang *et al.,* 2001; Marie *et al.,* 2002). The gold sensor surface is modified with a thiol- derivatised probe and a blocking thiol (Huang *et al.,* 2001; Mannelli *et al.,* 2003a; Herne and Tarlov, 1997). This kind of immobilisation chemistry has received increasing attention in the last years due to the possibility of forming a dense and compact surface coverage by the thiolated oligonucleotides. As demonstrated by Huang *et al.,* 2002, the thiolated probe alone could lie flat onto the surface because of non-specific interactions between the bases of the oligonucleotide and the gold surface. Moreover, the film of thiolated probes alone could not be compact because some gold regions could be uncovered. Due to this surface in-homogeneity the hybridisation efficiency is not high.

The immobilisation efficiency can be improved by the use of a blocking thiol such as MCH which attaches to the unoccupied gold regions replacing the non-specifically adsorbed probes. In this way, also a better orientation of the probe can be achieved with an increase in the hybridisation efficiency. In **Figure 1.15**, the immobilisation of thiolated probes followed by the use of blocking thiol (MCH) is shematically given. The probe has thiol functionalised group at the 5' end. In **Figure 1.16**, the diagram of frequency variations during the immobilisation steps of the thiolated probe directly onto the gold electrode of the quartz crystal is given as an example.

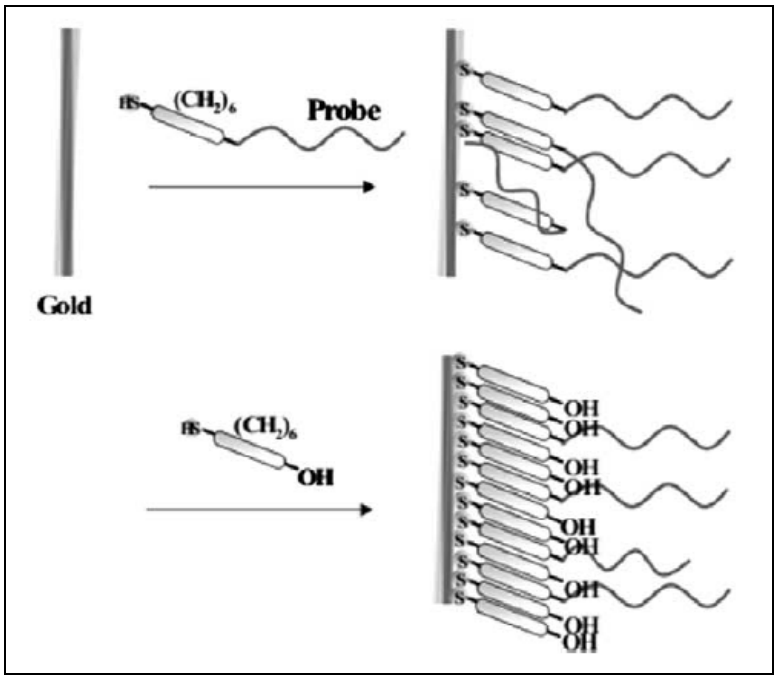

Figure 1.15. Immobilisation of thiolated probes followed by the use of blocking thiol (Mannelli *et al.*, 2003).

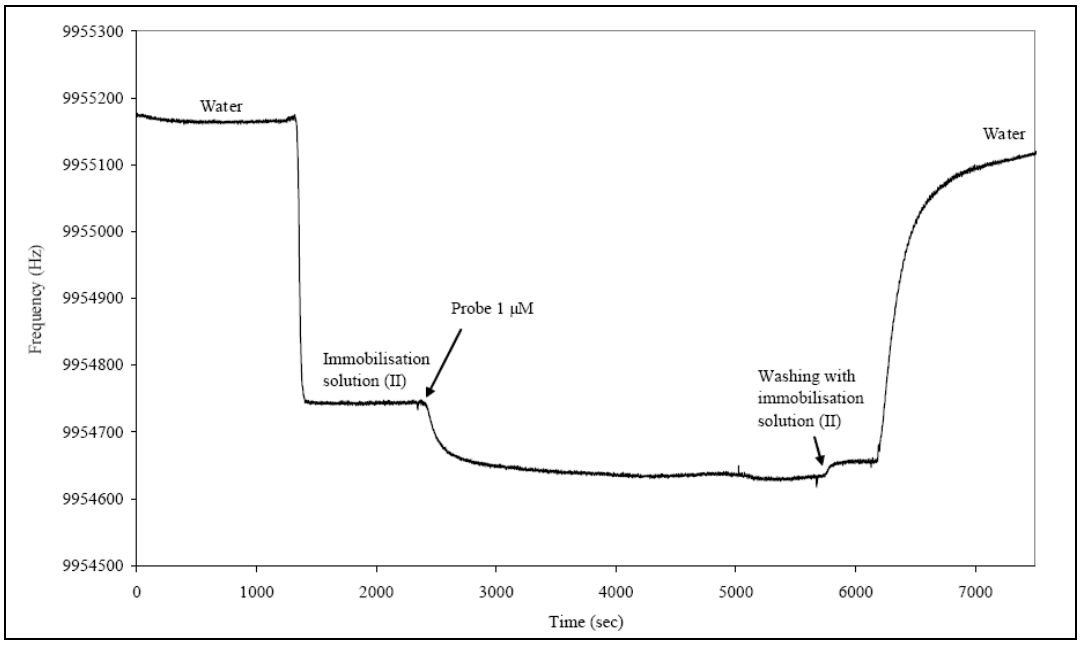

Figure 1.16. Frequency variations during the immobilisation of the thiolated probe directly onto the gold electrode of the quartz crystal (Tombelli *et al.*, 2005).

1.8.2.3.2.2. Immobilisation of probes by chemically modified surfaces (CMEs)

Plasma polymerisation is a specific type of process in plasma chemistry which involves reactions between plasma species or between plasma and surface species (Morosoff, 1990). The most featuristic aspect of plasma polymerisation technique is the thin polymer film, which can be prepared from almost any kind of organic vapors. This reaction may be caused in the polymerisation processes by high-energy ion bombardment. Unlike conventional organic polymers, plasma polymers do not consist of chains with a regular repeat unit but tend to form an irregular three-dimensional crosslinked network. The chemical structure and physical properties may be quite different from those of the conventional polymer, which is evenly derived from the same starting materials. Plasma-polymerised films are generally chemically inert, insoluble, mechanically tough, and thermally stable. Thus, these films have been used in a variety of applications such as permeably selective membranes, protective coatings, and electrical, optical, and biomedical films. **Figure 1.17** shows the potential applications of plasma polymerised films to sensor fabrication.

Surface modification of piezoelectric crystals for oligonucleotide immobilisation in which crystals are treated in glow-discharge systems were reported (Mutlu *et al.*, 1999). **Figure 1.18** represents the shematic drawing of a glow-discharge system. Several reactive or non-reactive plasmas can be created within the glow-discharge apparatus, which modify the surfaces by depositing films having functional groups. These, the so-called plasma-polymerised films covering the crystal surfaces, are extremely thin and homogenous coatings. They adhere strongly to the crystal and are highly resistant to chemical and physical treatments. Moreover, sensors produced using this method are more reproducible from sample to sample and exhibit lower noise than sensors made using conventional immobilisation methods. Ethylene diamine (EDA) plasma-polymerisation was applied to create amino groups on the quartz crystals and by using glutaraldehyde (GA) as a crosslinker or spacer arm, the single stranded oligonucleotides functionalised by amine groups at 5' end were immobilised on the crystals to prepare DNA biosensors by Duman *et al.*, 2003.

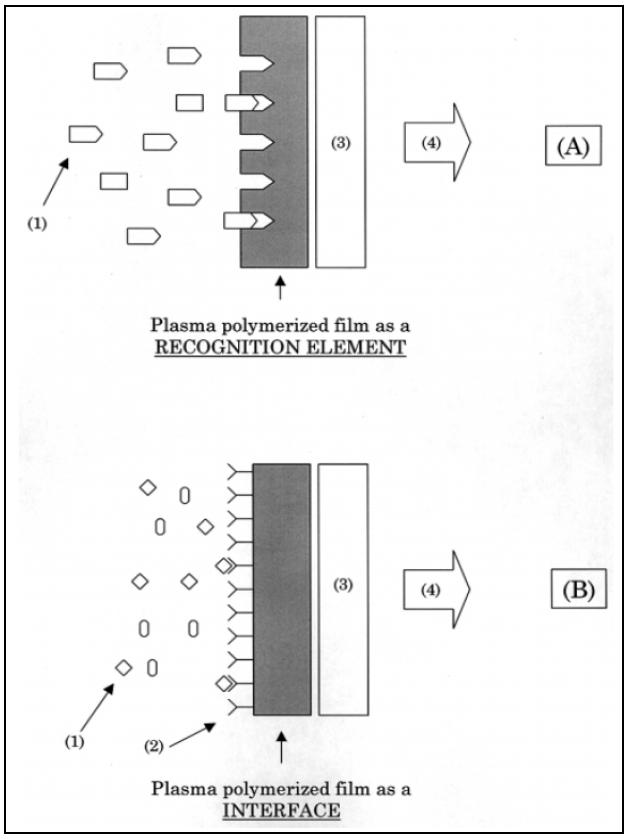

Figure 1.17. Typical architecture of chemical (A) and biological (B) sensors, and plasma polymerised films, which are incorporated as packaging, insulating, functional and interfacial materials. 1) analyte, 2) biological component as a recognition element, 3) transducer, 4) electronic signal (Hiratsuka and Karube, 2000).

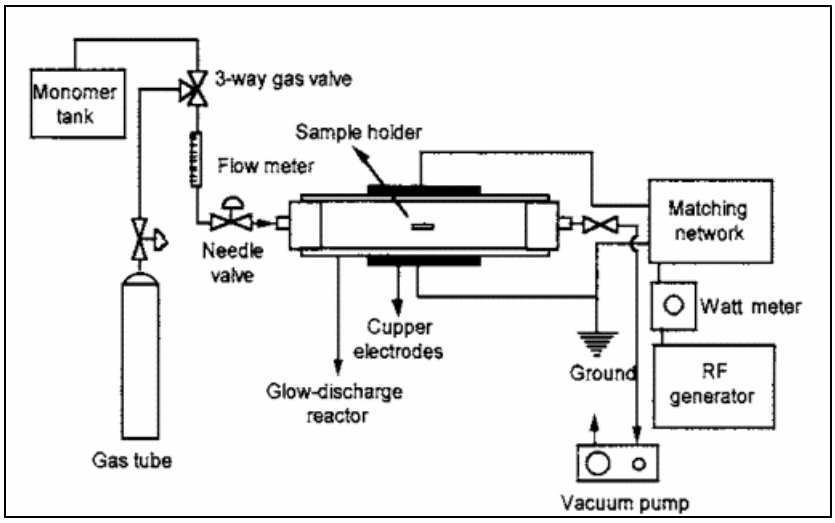

Figure 1.18. Shematic drawing of glow-discharge system (Mutlu *et al.,* 2002)

In the first step, the monomer, EDA, was allowed to flow through the reactor at a certain flow rate. The quartz crystals were exposed to the EDA plasma at an optimised glow-discharge power for a certain time. In the second step, the primary amine groups on the crystal surface created after the plasma-polymerisation of EDA were converted to aldehyde groups by treating the surfaces with gluteraldehyde (GA). It should be noted that a GA molecule has two aldehyde groups. At this treatment step, it was assumed that one aldehyde group on each GA is covalently bonded to amine group on the EDA treated crystal while the other aldehyde group stays free for the next treatment step.

1.8.2.3.3. Hybridisation measurements

Target sequences can be synthetic oligonucleotides that are complementary to the immobilised probes on biosensor's surface, longer sequences that includes complementary sequences of probes (PCR amplicons and real genomic DNA samples from genetically modified organisms). PCR products and genomic DNAs are double stranded in structure in which denaturation step is necessary before hybridisation with the single stranded probe. Hybridisations with synthetic oligonucleotides complementary to the immobilised probes on gold surface of QCM biosensor were mainly performed for the characterisation of the biosensor in terms of sensitivity, selectivity, reliability, reproducibility and stability. In addition,

calibration of the biosensor can be achieved by hybridising with different concentrations of target oligonucleotides. The amplicons obtained by PCR have a double helix structure and the two strands should be separated (denatured) to allow hybridisation with the probe immobilised on the sensor surface. To obtain a single strand from the amplicons, three approaches were followed mainly: thermal denaturation, magnetic separation of the strands and enzymatic digestion of one strand (Mannelli *et al.,* 2003).

1.9. Aim of the Study

In the last two decades, biosensors had a wide impact in clinical, food and environmental analysis. In particular, DNA-based biosensors, where a ssDNA probe is immobilised on the surface of a sensor, allow rapid, real-time monitoring of hybridisation with the target DNA sequence. The detection relies on the biorecognition between a probe immobilised on the sensing surface and the target sequence in solution (Mannelli *et al.,* 2005).

The application range of such a system is enormous. A very emerging environmental problem is the control of food containing genetically modified organisms (GMOs). GMOs are referred to as living organisms whose genomes have been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics, i.e. herbicide tolerance (Vollenhofer *et al.,* 1999) or resistance to viruses (Hails, 2000), antibiotics (Dröge *et al.,* 1998) and insects (Mariotti *et al.,* 2002).

On the need of new, fast and sensitive analytical methods for GMO screening, througout this study, development of a label free DNA-based detection method for genetically modified organisms was aimed.

Biosensors were prepared by immobilising the probe complementary to characteristic DNA sequences present in GMOs. The probe sequence was internal to the sequence of 35S promoter that is commonly inserted in the genome of the GMO regulating the transgene expression. Performance of different immobilisation procedures; thiol-gold

interaction with blocking thiol solution, functionalisation of ethylene diamine polymerised surfaces were investigated in terms of sensitivity, selectivity, stability and reproducibility.

The capacity of developed QCM-based DNA-biosensor in detection of CaMV 35S sequence in genomic DNAs of *pflp*-inserted transgenic tobacco plants was further tested by means of hybridisation reactions in the flow-cell system. The contribution of sonication and digestion of the DNAs on the performance of the system was investigated.

CHAPTER II

MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Chemicals

Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) and ethylenediaminetetraacetic acid (EDTA) were purchased from Duchefa. 6-mercapto-1-hexanol (MCH) and ethylenediamine (EDA) were from Fluka (Switzerland), and gluteraldehyde (GA) was from Aldrich (UK).

2.1.2. Synthetic Oligonucleotides

The 5'- functionalized synthetic oligonucleotides; -thiol, -amino -functionalised CaMV 35S probes and CaMV 35S target which was designed specifically for CaMV 35S promoter region was purchased from MVG Biotech AG (Ebersberg, Germany) and Thermo Electron Corporation (Waltham, MA, USA) in lyophilised form. The base sequence of the 5'- functionalised probe and of the complementary oligonucleotides (target; 25 bases) are reported below;

Thiolated CaMV 35S probe:
5' HS-$(CH_2)_6$-GGCCATCGTTGAAGATGCCTCTGCC 3'
Amined CaMV 35S probe:
5' H_2N- GGCCATCGTTGAAGATGCCTCTGCC 3'
Target (CaMV 35S): 5' GGCAGAGGCATCTTCAACGATGGCC 3'

2.1.3. Primer Sequences for CaMV 35S Promoter and NOS Terminator Regions

CaMV 35S promoter region specific sense and antisense primers which yields a 190 basepaired product and NOS terminator region specific sense and antisense primers which yields a 180 basepaired product were purchased from MVG Biotech AG (Ebersberg, Germany) and Iontek Inc. (Istanbul, Turkey) in lyophilised form. The base sequences of primers are reported below;

CaMV 35S sense: 5'-GCTCCTACAAATGCCATCA-3'

CaMV 35S antisense: 5'-GGATAGTGGGATTGTGCGTC-3'

TNos sense: 5' GAATCCTGTTGCCGGTCTTG 3'

TNos antisense: 5' TTATCCTAGTTTGCGCGCTA 3'

2.1.4. DNA Samples

Transgenic plant DNA samples were from *pflp* inserted (T6 and T10 lines) tobacco plants (*Nicotiana tabacum* cv. Samsun) and are kindly provided by T. Tuncer (2005). Wild type tobacco DNA (TC) were used as control. Plasmid map of *pflp* with P35S promoter and T-NOS terminator regions is given in **Appendix A**. Genomic DNAs were isolated by CTAB method and explained in section **2.2.3.1**.

2.1.5. QCM Crystals and Instrumentation

5 MHz, AT-cut Ti/Au polished piezoelectric quartz crystals (QCMs) were purchased from Maxtek Inc., USA. The quartz crystal was housed inside a methacrylate cell such that only one side of the crystal was in contact with the solution in the cell wall. The frequency was continuously recorded using a frequency counter Agilent counter Model 53181A 225MHz. The data is displayed on the main display screen and can be read directly by Excel©, Microsoft© on a computer connected to the frequency counter interface. In **Figure 2.1,** the flow-cell set-up of QCM frequency counter is photographed.

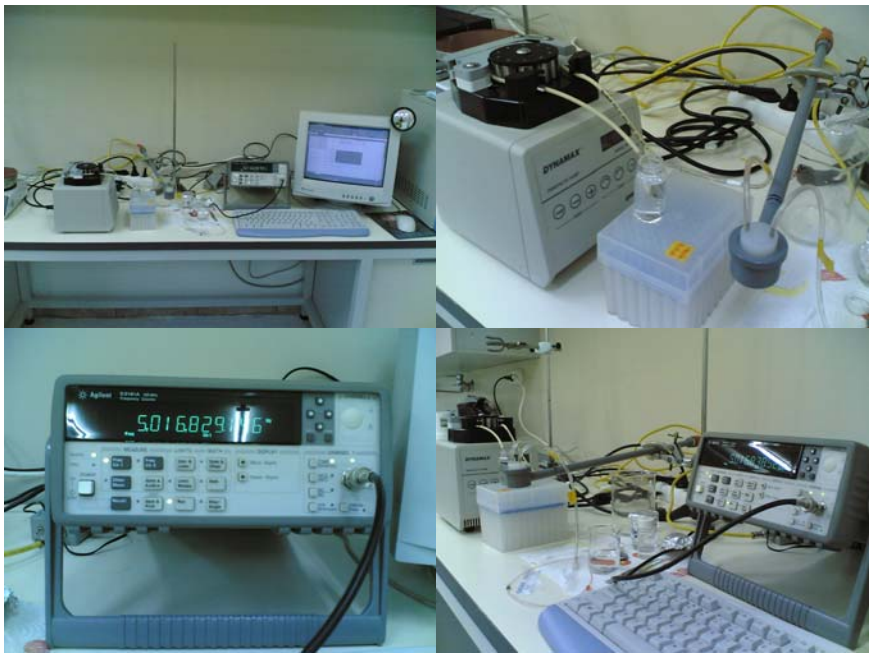

Figure 2.1. The flow-cell set-up of frequency counter

2.2. METHODS

2.2.1. Preparation of Synthetic Oligonucleotides

Sequences of synthetic oligonucleotides which are functionalised by thiol and amino groups were given in 2.1.1 and were prepared according to the manufacturer's recommendations. Lyophilised samples were dissolved in Tris.EDTA (pH:8.0) (**Appendix F**) buffer in order to reach a final 100 µM (pmole/µL) concentration and stored in (-) 20 °C until use.

2.2.2. Preparation of PCR Products (Amplified Regions from Plasmid DNA)

CaMV 35S and T-NOS PCR products were amplified from the vector pTJK136 which is a derivative of vector pTHW136. Vector pTJK136 harbors a gene coding for streptomycin/spectinomycin adenyl transferase which provides bacterial selection and an intron containing GUS gene and *npt-II* gene as plant selection markers for novel transgenic plant studies. GUS gene having an intron is localized between the CaMV 35S promoter and 3' nos terminator in T-DNA region. The map of pTJK136

plasmid DNA is given in **Appendix B**. Complete gene sequences of CaMV 35S promoter and NOS terminator are given in **Appendices C** and **D**, respectively, with depicted primer and promoter regions .

2.2.2.1. Preparation of Competent *E.coli* Cells

Competent *E.coli* cells were prepared according to the procedure of Inoue *et al.*, (1990). Firstly, the glycerol stock of *E.coli* strain DH5α cells were streaked on non-selective solid LB (**Appendix E**) plate and cultured overnigh at 37 °C. Then, ten to twelve colonies were isolated with a loop and inoculated to 250 ml of SOB medium (**Appendix E**) in a 2-liter flask. The culture was grown until an OD_{600nm} of 0.6 is reached at 18 °C, with vigorous shaking at 200-250 rpm. The grown culture were kept on ice for 10 minutes and then transferred to sterile centrifuge tubes. The centrifugation was done at 2500 x *g* for 10 minutes at 4 °C, in Sigma Laboratory Centrifuge Model 3K30, USA. The total pellet was resuspended in 80 ml of ice-cold TB (**Appendix E**) and incubated in ice for 10 minutes and then centrifuged at 2500 x *g* for 10 minutes at 4 °C. The pellet was resuspended in 20 ml of TB and DMSO (Dimethyl sulfoxide) was added with gentle swirling to a final concentration of 7 %. After incubation in ice for 10 minutes, the cell suspension was dispensed by 0.2 ml into sterile eppendorf tubes and immediately chilled in liquid nitrogen. Finally the frozen competent cells were stored at (-) 80 °C.

2.2.2.2. Bacterial Transformation with pTJK136 Plasmid DNA

The bacterial transformation of competent *E.coli* strain DH5α with pTJK136 plasmid DNA was performed by heat shock method according to Maniatis *et al.,* (1989) with minor modifications. An eppendorf tube of competent cells from -80 °C stock was freeze-thawed on ice. 100 µL competent *E.coli* and 2 µL plasmid DNA (0.3 µg/µL) were added in a sterile eppendorf tube and shaked gently to mix. After 1 hour of ice incubation, the mixture was kept in 42 °C in a water-bath incubator for only 90 seconds and then immediately put onto ice again for 5 minutes. Then 900 µL LB medium was added onto the mixture and shaken for 1 hour at 37 °C at 100 rpm. Then, 200 µL was spread on 100 mg/L streptomycin and 50 mg/L spectinomycin

containing solid LB plate which would sellect *E.coli* cells transformed with pTJK136 plasmid DNA. The rest of the mixture was centrifuged in a microfuge at 3000 rpm for 3 minutes and 800 µL of the supernatant which was mostly LB was discarded and the pellet was dissolved in remaining LB and spreaded on solid LB plate containing the same antibiotics. The plates were incubated at 37 °C overnight. A single colony from the plate was grown in liquid LB supplemented with antibiotics streptomycin and spectinomycin. Freshly grown culture was used for miniprep plasmid DNA isolation.

2.2.2.3. Miniprep Plasmid DNA Isolation

Plasmid DNA isolation (miniprep) for *E.coli* was performed by alkaline lysis method according to Sambrook *et al.,* (1989). A single colony of pTJK136 plasmid containing *E.coli* was grown overnight in liquid LB at 37 °C, at 200 rpm. Bacterial culture was aliquoted into 1.5 mL eppendorf tubes and centifuged 1 minute at 12000 rpm in a bench-top eppendorf centrifuge at 4 °C.

Supernatants were removed and the bacterial pellets were left as dry as possible. The pellets were resuspended in ice-cold 100 µL of Solution I (**Appendix F**) by vigorous vortexing. Then, 200 µL of freshly prepared Solution II (**Appendix F**) was added and the contents were mixed slowly by inverting the tubes 6-8 times and stoved on ice for 5 minutes. After incubation of eppendorf tubes on ice, 150 µL of ice-cold Solution III (**Appendix F**) was added and vortexed for 2 seconds and stored on ice for another 5 minutes. Equal volume of chloroform:isoamyl alcohol (24:1)(v/v) was added and mixed by vortexing. Then, centrifuged at 12000 rpm for 5 minutes in order to separate the phases. After centrifugation, aqueous (upper) phase was taken into a new sterile eppendorf tube. dsDNA was precipitated with 2X volumes of chilled 96 % ethanol and centrifuged for 5 minutes at 12000 rpm. Supernatant was removed and 1 ml of 70 % ethanol was added in order to wash the plasmid DNA. After washing step, the tubes were centrifuged at 12000 rpm for 2 minutes and the supernatants were removed and the pellets were air dried. The pellets were dissolved in 50 µL TE (**Appendix F**) containing 20 µg/L Rnase A and incubated at 37 °C for at least half an hour in order to get rid of RNAs. Finally, the tubes were centrifuged at

12000 rpm for 2 minutes or more and the supernatant were removed and the DNA pellets were dissolved in 30-50 µL TE and stored at (-) 20 °C. pTJK136 plasmid DNA isolated by alkaline lysis miniprep method was used for amplification of selected regions of CaMV 35S promoter and NOS terminator genes.

2.2.2.4. PCR Conditions for CaMV 35S Promoter Region Primers

PCR was carried out in a total reaction mixture of 50 µL. All of the components of PCR were kept in ice before usage. Optimised PCR conditions for CaMV 35S promoter region, *5'-GCTCCTACAAATGCCATCA-3'* sense and *5'-GGATAGTGGGATTGTGCGTC-3'* antisense primers are listed as follows;

	CaMV 35S primers
dH_2O	35.5 µL
10X Reaction Buffer	5 µL
25 mM $MgCl_2$	3.5 µL
2.5 mM dNTP	2.5 µL
Primer Sense (25 pmol/µL)	1 µL
Primer Antisense (25 pmol/µL)	1 µL
Taq Polymerase (5 u/µL)	0.5 µL
DNA (1/10 dilution of miniprep isolation)	1 µL
Total Volume	50 µL

Cycling program of PCR for CaMV 35S primers were reported as follows;

	CaMV 35S PCR cycle	
Initial Denaturation	95°C 5 minutes	
Denaturation	95°C 30 seconds	
Annealing	54°C 30 seconds	35 cycles
Extension	72°C 60 seconds	
Final Extension	72°C 5 minutes	

2.2.2.5. PCR Conditions for T-NOS Terminator Region Primers

PCR was carried out in a total reaction mixture of 50 µL. All of the components of PCR were kept in ice before usage. Optimised PCR conditions for T-NOS terminator region, *5'-GAATCCTGTTGCCGGTCTTG-3'* sense and *5'-TTATCCTAGTTTGCGCGCTA-3'* antisense primers are listed as follows;

	T-NOS primers
dH_2O	33.7 µL
10X Reaction Buffer	5 µL
25 mM $MgCl_2$	2.5 µL
2.5 mM dNTP	3.3 µL
Primer Sense (25 pmol/µL)	2 µL
Primer Antisense (25 pmol/µL)	2 µL
Taq Polymerase (5 u/µL)	0.5 µL
DNA (1/10 dilution of miniprep isolation)	1 µL
Total volume	50 µL

Cycling program of PCR for T-NOS primers were reported as follows;

	T-NOS PCR cycle	
Initial Denaturation	95°C 4 minutes	
Denaturation	94°C 30 seconds	
Annealing	55°C 45 seconds	30 cycles
Extension	72°C 90 seconds	
Final Extension	72°C 7 minutes	

2.2.2.6. Agarose Gel Electrophoresis of PCR Products

PCR products were run on 1 % agarose gel buffered with 1X TAE. 3 µL of ethidium bromide (10 mg/ml) was added to 50 ml of agarose gel solution. The microwave oven-melted gel solution was poured into an electrophoresis gel tray and the comb was placed to form wells. After removal of the comb and placing of the gel in 1X TAE buffer-filled electrophoresis tank, PCR samples and molecular weight size markers (100 bp DNA ladder, MBI Fermentas) were loaded into wells by mixing with 6X loading buffer (Fermentas) at a final concentration of 1X. Power supply was adjusted to 75-100 V and the gel was run for about 1 hour. Then the bands were visualised under UV light and documented in Vilbert-Lismart gel documentation system for concentration determination of PCR products.

2.2.2.7. Gel Extraction of PCR Products

The bands of PCR products that were run on 1 % agarose gel were visualised under UV light and the bands corresponding to 196 bp product for CaMV 35S and 180 bp product for T-NOS were cut with a sterile scapel and transferred into sterile eppendorf tubes. PCR products were extracted from the agarose gel by using Gel Extraction Kit of Genemark according to the manufacturer's recommendations. In order to obtain enough of PCR products for the experiments, consecutive extraction procedure was done.

2.2.2.8. Phenol/Chloroform/Isoamylalcohol Extraction of PCR Products

After PCR was performed either with CaMV 35S primers or with NOS primers, some of the PCR product containing tubes were extracted with phenol-chloroform-isoamylalcohol (pH:7.5-8.0) instead of gel extraction in order to get rid of PCR elements in the reaction mixture and get a clear PCR product as a resultant. Equal volume of phenol-chloroform-isoamylalcohol solution was added to the reaction mixture in PCR tube and mixed gently. Then centrifugation was done in a microfuge at high speed (15000 rpm) and the top aqueous phase containing the PCR products was transferred to a new sterile tube and ethanol precipitation was done. The pellet was then dissolved in TE buffer or sterile deionized water. PCR product concentration was determined densitometrically from the photograph of a sample on an agarose gel which was run with a known concentration of molecular weight marker.

2.2.2.9. PCR Product Concentration Determination

Spectrophotometric analysis was done in order to determine the concentration of PCR products diluted in Immobilisation Solution (**Appendix G**). Spectrophotometric reading was done at A_{260nm} after auto-zeroing with the blank solution (immobilisation solution). The measured value at A_{260nm} was recorded.

The concentration of pure double-stranded DNA with an A_{260nm} of 1.0 is 50 µg / ml. The PCR products were also double-stranded. Thus, we can use the following formula **(1)** to determine the concentration of PCR products.

Unknown (µg / mL) / Measured A_{260nm} = 50 (µg / mL) / 1.0 A_{260nm} **(1)**

Since there is a linear relationship between absorbance and DNA concentration, we can use some simple algebra and reformulate **(1)** to **(2)** as follows:

Unknown µg / mL = 50 µg / mL x Measured A_{260nm} x dilution factor **(2)**

Also, densitometric analysis was done for the concentration determination of PCR products. The bands of PCR products that was loaded at a certain volume on agarose gel visualized under UV light were documented by Vilbert-Lismart gel documentation system. The concentration of bands were determined quantitatively by comparing with a known concentration of band in the molecular weight marker. Finally, total concentration of PCR products in nanograms was determined in the stock solution.

2.2.2.10. Denaturation of PCR Products

The amplicons obtained by PCR have a double helix structure and to allow the hybridisation with the probe immobilised on the sensor surface, the two strands should be denatured. To obtain single strand from the amplicons (PCR products) thermal denaturation procedure was followed. The PCR products were denatured by heating at 95 °C for 5 minutes and then cooled in (-) 20 °C for 2 minutes. The denatured samples were immediately introduced into the flow-cell of QCM for the measurement of frequency change upon hybridisation.

2.2.3. Preparation of Real Samples from Plant Genomic DNAs

Transgenic plant DNA samples were from *pflp* (**Appendix A**) inserted (T6 and T10 lines) tobacco plants (*Nicotiana tabacum* cv. Samsun) which were reported as a sample containing the target sequences of promoter P35S DNA and terminator T-Nos DNA (Tuncer, 2006). Wild type tobacco DNA was used as a control.

2.2.3.1. Plant Genomic DNA Isolation by CTAB Method

The genomic DNAs from fresh leaf tissues were isolated by using the CTAB method described by Doyle and Doyle (1990). 1-2 grams of plant leaf tissue was ground to powder in liquid nitrogen in a mortar and pestle. The powder was directly scraped into preheated (65 °C) 10 ml of CTAB isolation buffer [2 % CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 0.2 % β-mercaptoethanol, 20 mM EDTA, 100 mM Tris.HCl (pH:8.0)], mixed gently and incubated at 60 °C for

30-45 minutes with occasional swirling. The solution then cooled and extracted with chloroform:isoamylalcohol (24:1 (v/v)) and mixed gently and throughly. Centrifugation was done at 10000 rpm for 10-15 minutes in order to concentrate phases. The upper, clear aqueous phase was removed with a wide-bore pipette and transferred to a clean centrifuge tube. 2 / 3 volume of cold isopropanol was added to the solution and mixed gently to precipitate the nucleic acids. Centrifugation was done at 10000 rpm for 5 minutes in order to get the DNA pellet. Then the pellet was washed with 70 % ethanol and air dried. After removal of all ethanol, the pellet was resuspended in 1 ml TE buffer (**Appendix F**) containing a final concentration of 20 µg/mL RNAse A and incubated at 37 °C for 30 minutes. After incubation, the samples were diluted with two volumes of TE buffer and ammonium acetate (pH:7.7) was added to a final concentration of 2.5 M then, the samples were mixed gently and 2.5 volumes of cold absolute ethanol was added. After centrifugation at 12000 x *g* for 5 minutes, the pellets were air dried and resuspended in appropriate amount of TE buffer.

The concentration of genomic DNAs were determined by spectrophotometric analysis by measuring absorbances at 260 nm.

2.2.3.2. Digestion of Genomic DNAs with Restriction Enzymes (REs)

Digestion of isolated genomic DNAs were performed at different incubation times, i.e; 2, 8 hours or overnight (~16 hours). 5 µg of isolated genomic DNA was digested with BamH1 and BamH1/HindIII (2 units RE / µg DNA) at given incubation time periods, at 37 °C. BamH1 cuts T-DNA of *pflp*-inserted from one side and frees P35S region flanking at one end. Digestion with both BamH1 and HindIII totally frees P35S region in T-DNA. The restriction enzyme map of T-DNA region of *pflp* plasmid is given in **Appendix A**. Digestion products were run on agarose gel electrophoresis in order to obtain the digestion profiles of the DNAs with one and two restriction enzymes. Components of reaction mixture of restriction enzyme digestions with BamH1 were as follows;

	Wild type tobacco	T10-line
Sterile dH_2O	(79-X) µL	(79-X) µL
10X Buffer Y/Tango® (2X final)	20 µL	20 µL
DNA (5µg total)	X µL	X µL
Bam H1 (10u/µL)	1 µL	1µL
Total volume	100 µL	100 µL

Components of reaction mixture of restriction enzyme digestions with BamH1 and HindIII were as follows;

	Wild type tobacco	T10-line
Sterile dH_2O	(78-X) µL	(79-X) µL
10X Buffer Y/Tango® (2X final)	20 µL	20 µL
DNA (5µg total)	X µL	X µL
Bam H1 (10u/µL) HindIII (10u/µL)	1 µL 1 µL	1µL 1µL
Total volume	100 µL	100 µL

2.2.3.3. Sonication of Genomic DNAs

Besides digestion with restriction enzymes, isolated genomic DNAs were distrupted with sonication. 5 µg DNA in 100 µL ultrapure water in PCR tubes were incubated on ice during the sonication. Sonicator probe was in 2 mm diameter and the output energy was 2 Watts. The frequency was kept constant at 20 kHz. Different incubation times (0 to 90 seconds with 15 seconds intervals) were tried in order to get fragmented DNA and results were checked with agarose gel electrophoresis.

2.2.4. QCM Device and Basis of Piezoelectrical Measurement

5 MHz quartz crystals (Maxtek Inc., USA) with gold electrodes evaporated on both sides (**Figure 2.2**) were used for piezoelectrical measurements of hybridisations of synthetic oligonucletides, denatured PCR products and genomic DNAs of real

samples (GMO). The sensing surface of crystals (~ 0.40 cm^2) were immobilised with single stranded oligonucleotides (probes) designed from promoter CaMV35S gene sequence via appropriate immobilisation procedure. Target sequences in hybridisation buffer were passed through the QCM flow-cell set-up at a certain flow rate (i.e. 50 - 100 µL / min) and hybridisation of complementary strands were monitored for about 20 minutes. Basal resonance frequency values were recorded before and after the hybridisation event. The frequency shift ($\Delta F = F_B - F_A$) between the initial (F_A) and final (F_B) stable frequency values were reported and the amount of hybridised target was calculated according to the Sauerbrey equation (**Section 1.8.2.3.1.(I)**). In all experiments, the single stranded probe was regenerated by 1 minute treatment with 1 mM HCl allowing a further use of the sensor. All the experiments were performed at room temperature (~ 25 °C).

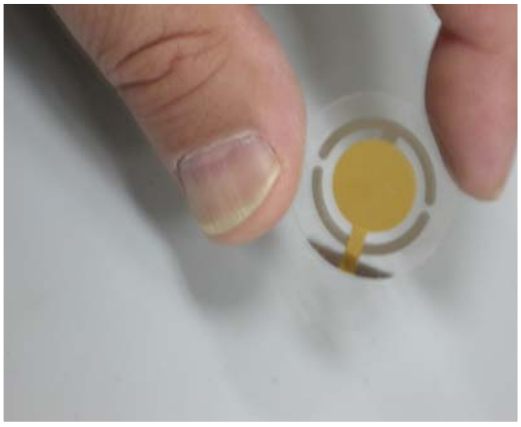

Figure 2.2. 5 MHz quartz crystals (Maxtek Inc., USA) with gold electrodes evaporated on both sides.

2.2.5. Cleaning of Crystals

The gold electrode surface of quartz crystal microbalances (5 MHz-QCMs, Mastek Inc., USA) were cleaned either with a boiling solution of H_2O_2 (30 %), NH_3 (30 %) and ultrapure water (ELGA) in a 1:1:5 ratio or with hot Piranha solution (30 % H_2O_2:H_2SO_4/1:3) for 10 minutes or more and then throughly rinsed with ultrapure (ELGA) water and absolute ethanol. They were used immediately afterwards or waited in absolute ethanol until use.

2.2.6. Preparation of Solutions

The compositions of immobilisation solution and hybridisation buffer used in piezoelectric experiments are given in **Appendix G** (Mannelli *et al.,* 2005). All buffers and solutions used in the experiments were prepared with ultrapure water (ELGA) and autoclaved at 121 °C, at 1.5 atmospheric pressure for 20 minutes.

2.2.7. Immobilisation Procedures of Probes on QCM Sensing Surface

Probe immobilisation is the fundamental step in DNA-based biosensor development. In this study, two different functionalised-probe types were used for the immobilisation of the 5 MHz - quartz crystal gold surfaces. One of them was the thiol-derivatized probe and the other was the amino-derivatized probe. Two different immobilisation procedures were employed for those probe types; thiol-derivatised probe and blocking thiol procedure (Mannelli *et al.,* 2005) and amino-derivatised probe immobilisation by forming ethylenediamine monolayer on gold surface by glow-discharge method (Mutlu *et al.,* 1999).

2.2.7.1. Thiol-probe Immobilisation

Thiol-derivatised probe and blocking thiol procedure was performed according to Mannelli *et al.,* 2005, with minor modifications. That procedure was also modified from the study of Herne and Tarlov, 1997. The method included the direct chemisorption of DNA probes onto transducer surfaces (quartz crystal surface) based on the formation of gold-thiol bonds. The gold sensor surface was modified with thiol-derivatised probe (oligo - C_6 - SH) solution by simply dipping the crystal in 1 ml of 2 μM thiol-derivatised probe prepared in 1 M Immobilisation Solution (pH:3.8) (**Appendix G**). Then, the crystal was either treated with a 1 ml of 1 mM blocking thiol (6 – mercapto – 1 – hexanol, MCH) solution for 1 hour or not. Before immobilisation of the thiolated probe, basal frequency of the crystal was measured in immobilisation solution. Then, the cleaned crystal surface was treated with thiolated probe prepared in immobilisation solution. After washing with immobilisation solution and ultrapure water (ELGA), blocking thiol (MCH, 1 mM in ultrapure

water) solution was added to the cell and the reaction was allowed to proceed for 1 hour. After washing with ultrapure water, basal frequency of the crystal in immobilisation solution was measured again. Frequency values were recorded when a stable frequency signal was obtained. The frequency difference between the values before and after the immobilisation process was used in Sauerbrey equation to determine the amount of mass deposited on the crystal surface by immobilised thiolated probe and blocking thiol.

2.2.7.2. Amino-probe Immobilisation

Amino-derivatised probe immobilisation on quartz crystal surface was achieved by treating in a glow-discharge system (Pico RF, DIENER Electronic, Germany). It generates a radio-frequency of 13.6 MHz. The monomer tank was connected to the reactor through a flow meter and a needle valve. Ethylene diamine (EDA) was used as the active monomer and was plasma polymerized in the glow-discharge reactor to create amine-like active groups on the quartz crystal surface for further treatments needed for the immobilisation of amino-derivatised probe. The glow-discharge reactor was evacuated to 10^{-1} mbar. The monomer was allowed to flow through the reactor at a flow rate of 30 ml/min. The quartz crystals were exposed to EDA plasma for 5 minutes at a glow-discharge power of 20 Watt. The conditions were adjusted from the optimised values of EDA plasma polymerisation in glow-discharge system by Mutlu *et al.,*1999, Saber *et al.,* 2002, Duman *et al.,* 2003. The modifications on the quartz crystal surface was followed by measuring the resonance frequency shifts before and after the plasma-polymerisation process. The primary amine groups created on the crystal surface after plasma-polymerisation of EDA were further converted to aldehyde groups by treating the surfaces with 2.5% gluteraldehyde (GA) in carbonate buffer (pH:7.4) at room temperature for 16 hours. At this treatment step, it was assumed that one aldehyde group of gluteraldehyde was covelently bonded to amine group on the EDA treated surface and the other aldehyde group stayed free for the immobilisation of amino-derivatised probe. Proceeding reactions in ethylene diamine plasma polymerisation and gluteraldehyde activation steps are shown in **Figure 3.16** (Akdoğan *et al.,* 2006).

2.2.8. Optimisation of Immobilisation Parameters

Achievement of a functional immobilisation procedure is a challenge in DNA-based biosensor development. Probe concentration, immobilisation time and method were further optimised for thiol-probe in order to obtain an effective immobilisation system for a better characterization of the QCM-based DNA biosensor.

2.2.8.1. Probe Concentration

Effective probe concentration was studied with both thiol and amino-derivatised probes by using concentrations of 0.5 μM, 1μM, 2μM, 5μM and 10μM during immobilisation of the quartz crystal surfaces. 2 hours immobilisation time was kept constant. QCMs were directly dipped into the prepared solutions of probes. Before and after immobilisation process, surfaces were washed with immobilisation solution and resonance frequency values (F_A; before, F_B; after) were recorded. Frequency shifts ($\Delta F = F_B - F_A$) due to deposition of the probes on the QCM surfaces were measured in order to obtain the effective probe concentration.

2.2.8.2. Probe Immobilisation Time

Effective time for immobilisation of thiol and amino - derivatised probes on quartz crystal surfaces were also studied. For that, three different immobilisation durations (2 h, 8 h and 16 hrs) were tested at a constant 5 μM probe concentration. Resonance frequency values were recorded before (F_A) and after (F_B) the immobilisation process and the better immobilisation time was decided according to the calculated frequency shift ($\Delta F = F_B - F_A$) upon probe deposition on quartz crystal surfaces.

2.2.8.3. Immobilisation Method

Immobilisation of thiol and amino - derivatised probes on QCM surfaces either by dipping of the crystals directly into the prepared solutions of probes in immobilisation solution II or by passing the probe solution supplied from an eppendorf tube - resorvoir through the QCM surfaces via a flow - cell set - up was

investigated. Dipping of the crystals were either done by directly soaking them in 1 ml of probe solution in appropriate caps for immobilisation at 4 °C or by adding 500 µl probe solution onto the crystals placed into the methacrylate cell - house of the frequency counter system.

2.2.9. QCM-based Hybridisation Experiments

Hybridisations with the target synthetic oligonucleotides, PCR products and genomic DNAs of real samples were done with the QCM sensors that were immobilised by thiol - derivatised P35S probes. All the experiments were performed at room temperature (~ 25 °C) with the QCM flow - cell system.

2.2.9.1. Characterization of the QCM Sensor

In order to characterise the QCM sensor, the synthetic oligonucleotide Target P35S (1 µM) complementary to the immobilised probe P35S was used. Firstly, hybridisation buffer was flowed through the QCM surface for basal resonance frequency determination. Then, hybridisation with the target oligonucleotide was performed by flowing the target oligonucleotide solution in hybridisation buffer through the flow - cell system. Later, hybridisation buffer was flowed again for the removal of unbound oligonucleotides. In all steps, monitoring continued till a steady frequency value was reached. The frequency shift ($\Delta F = F_B - F_A$) was reported as the difference between the final value (F_B) and the value displayed before (F_A) the hybridisation reaction. The single stranded probe immobilised on QCM surface was regenerated by treating 1 minute with 1 mM HCl for further use of the sensor. Frequency change versus time graph for the hybridisation of target oligonucleotide with immobilised probe on QCM electrode was plotted.

2.2.9.2. Calibration of the QCM Sensor

Calibration of the QCM sensor was performed by hybridising the P35S - immobilised probe on electrode surface with changing concentrations of synthetic Target P35S oligonucleotides from 0.1 µM to 10 µM prepared in hybridisation

buffer. Each target concentration was studied on separate QCMs immobilised with 2 μM thiolated P35S probe in immobilisation solution for 2 h, following a blocking step with 1 mM mercaptohexanol (MCH) for 1 h. The profile was drawn as ΔF (Hz) versus target P35S concentration (μM). Effective target concentration was reported. The specificity of the system was tested with non - complementary (Target T - NOS) oligonucleotide.

2.2.9.3. Synthetic Target Oligonucleotides

Hybridisations with the determined (calibrated) concentrations of synthetic target oligonucleotides were performed in order to characterise the analytical performances of biosensors in stability, specificity, reproducibility and stability. Thiolated and amined P35S probes were immobilised and analysed separately on different QCM - based biosensors in the flow - cell set - up at a flow rate of ~ 50 μl / min. Regeneration of the biosensors was done with 1 minute treatment of 1 mM HCl between consecutive hybridisation events. All reactions were performed at room temperature.

2.2.9.4. PCR Products

The best conditions of immobilisation, probe concentration and contact time were adopted for the analysis of PCR products. P35S PCR products were phenol / chloroform / isoamyl alcohol extracted after PCR. Serial diluted samples of PCR products were tested with separate biosensors after the heat denaturation step. Frequency shift versus time graph for the hybridisations of changing concentrations of PCR products with the immobilised probes was plotted. The specificity of the system was tested with T - NOS PCR products.

2.2.9.5. Real DNA Samples

Optimised immobilisation conditions were also used for the analysis of digested and sonicated genomic DNAs of real samples; wild type and *pflp* inserted T10 - lines of tobacco plants (*Nicotiana tabacum* cv. Samsun) (Tuncer, 2006). Sonicated and

restriction enzyme digested samples of genomic DNAs were serially diluted with hybridisation buffer and hybridisations with the immobilised probes were performed on different QCM biosensors. Frequency shift versus time graph for the hybridisations of changing concentrations of real samples with the immobilised probes was plotted. The specificity of the system was controlled with the wild type genomic DNA of tobacco (*Nicotiana tabacum* cv. Samsun).

CHAPTER III

RESULTS & DISCUSSION

3.1. Primer and Probe Sequences for CaMV35S and T-NOS

Almost all commercially developed transgenic crop plants contain either the CaMV 35S promoter or the T-NOS terminator. A study conducted in 1997 concluded that either the CaMV 35S promoter or the T-NOS terminator sequences were present in 27 of 28 EU-approved genetically modified crops (Hemmer, 1997). So, the ability to detect these elements will allow detection of the vast majority of GMO plant material. Also, validated detection methods are based mainly on these sequences (Spoth and Strauss, Promega Corporation).

In this study, the primer sequences that are used for CaMV 35S promoter and T-NOS terminator regions produce 196 bp and 180 bp PCR products, respectively. The probe sequences are internal to the amplified product of CaMV 35S and T-NOS using the primers reported (**Appendices C, D**).

These primer and probe sequences were also previously studied in GMO detection systems of other working groups (Minunni *et al.,* 2001a,b; Mariotti *et al.,* 2002; Mannelli *et al*., 2003a,b, 2005; Giakoumaki *et al*., 2003; Meriç *et al.* 2004).

3.2. Preparation of PCR products and Genomic DNA Samples

3.2.1. PCR Products

pTJK136 plasmid DNA (**Appendix B**) was used as a template for both CaMV 35S and T-NOS primers in PCR analysis. Before performing PCR procedures, amplification of pTJK 136 plasmid DNA was achieved by transforming the plasmid DNA to competent *E.coli* cells. Transformed bacterial colonies were selected on Streptomycin (50 mg/L) and Spectinomycin (50 mg/L) containing LB agar medium and amplified by culturing in liquid LB medium. Agarose gel electrophoresis result for miniprep isolated pTJK136 plasmid DNA is illustrated in **Figure 3.1**.

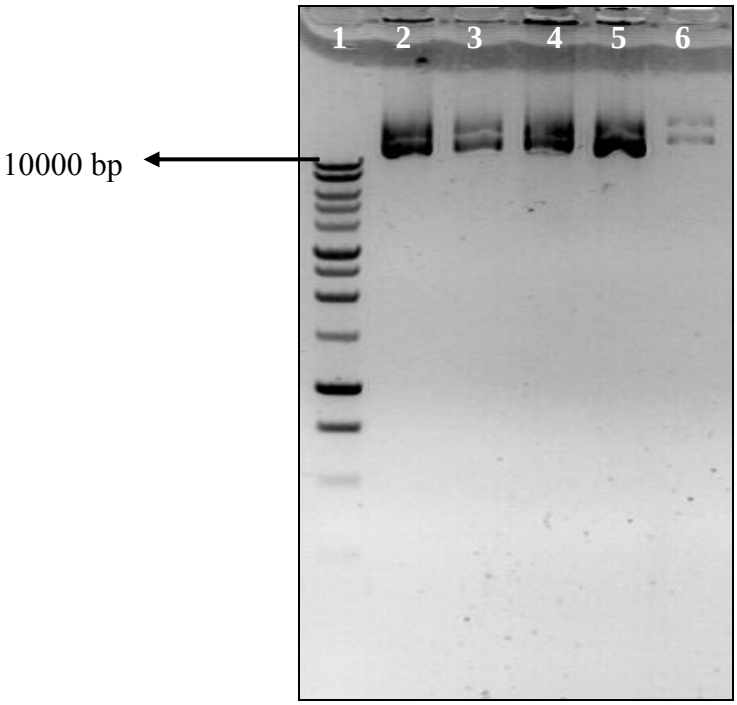

Figure 3.1. 1% Agarose gel electrophoresis (in 1X TAE buffer) of miniprep isolated pTJK 136 plasmid DNA. Lane1;1kb DNA ladder (6µL), (G571A-Promega); Lanes 2-5; pTJK 136 plasmid DNA (5µL each); Lane 6; 1/10 dilution of pTJK 136 plasmid DNA in lane 2 (5µL).

PCR analysis is done for both CaMV 35S and T-NOS primers and agarose gel electrophoresis is performed as a post PCR analysis in order to confirm the presence of the expected sized PCR products with respect to standard molecular markers. The corresponding CaMV 35S and T-NOS PCR products bands are shown in **Figure 3.2**.

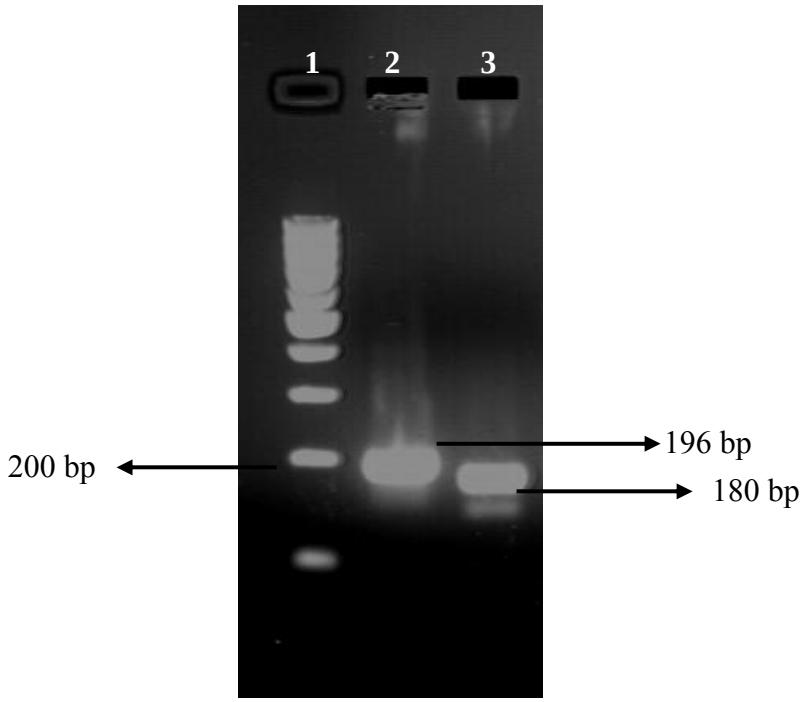

Figure 3.2. 1% Agarose gel electrophoresis (in 1X TAE buffer) of T-NOS and CaMV 35S PCR products amplified from pTJK 136 plasmid DNA. Lane 1; 100bp DNA ladder (MBI Fermantas), Lane 2; CaMV 35S PCR product (196bp), Lane 3; T-NOS PCR product (180bp)

3.2.2. Wild Type and *pflp*-inserted Tobacco Genomic DNAs

Genomic DNAs of control and *pflp* gene inserted transgenic tobacco (*Nicotiana tabacum* cv. Samsun) plants were isolated with CTAB method and further extracted with phenol/chloroform in order to have a purified DNA isolates. Wild type and *pflp* gene inserted tobacco plants were kindly provided by T. Tuncer. Agarose gel electrophoresis of CTAB isolated and double phenol extracted genomic DNAs of wild type tobacco, T6 and T10 lines of pflp-inserted tobaccos is illustrated in **Figure 3.3** and **3.4**, respectively.

The concentration of the DNAs were determined by spectrophotometric analysis. Absorbance values at 260nm and 280 nm were recorded and concentrations in µg/ml and purity of the DNAs were calculated. Also densitometric analysis was performed for the genomic DNA bands on agarose gel by comparing the intensity of the fluorescence emitted by ethidium-bromide stained DNA bands relative to a DNA standard of known concentration.

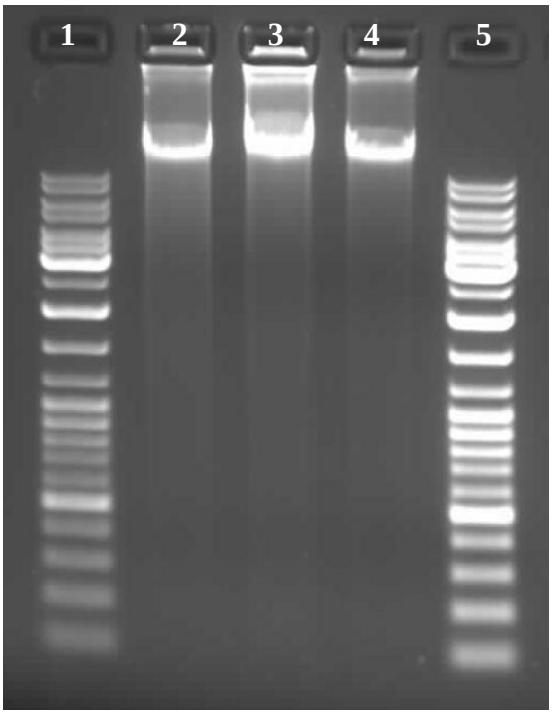

Figure 3.3. 1% Agarose gel electrophoresis of CTAB isolated genomic DNAs. Lanes1&5; Gene Ruler™ DNA ladder mix (MBI Fermentas-#SM0331) (6 and 12µL), Lane2; wild type tobacco DNA, Lane3; T6-line pflp-inserted tobacco DNA, Lane4; T10-line pflp-inserted tobacco DNA.

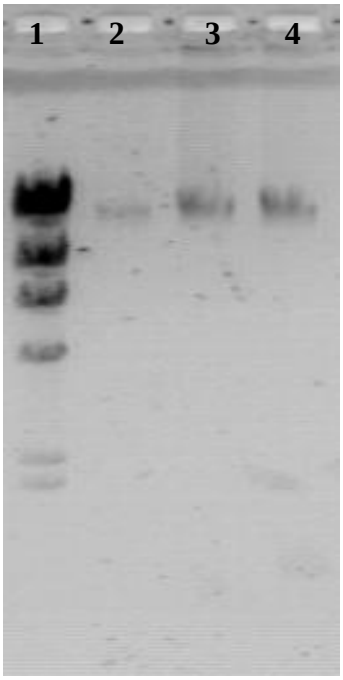

Figure 3.4. 1% Agarose gel electrophoresis of CTAB isolated, double phenol extracted genomic DNAs. Lane1; Lambda DNA/HindIII Marker,2 (MBI Fermentas-#SM0101), Lane2; wild type tobacco DNA, Lane3; T6-line pflp-inserted tobacco DNA, Lane4; T10-line pflp-inserted tobacco DNA.

3.2.2.1. Digestion of Genomic DNAs

A total of 5µg DNA in reaction tubes were digested with either one restriction enzyme; BamH1 or two restriction enzymes; BamH1 and HindIII. BamH1 cuts the inserted T-DNA from 5' end of P35S promoter region and frees 5' end flanking. HindIII with BamH1 totally removes the P35S promoter region in the matrix (**Figure 3.5**). Different incubation times (2, 8, or 16 hours) were applied for both of the digestion reactions at 37°C. The presence of accidental recognition sequences for both BamH1 and HindIII were controlled within P35S promoter sequence region (**Appendix C**) and found to be absent.

5'...G▼GATCC...3' BamH1 recognition site
3'...CCTAG▲G...5'
and
5'...A▼AGCTT...3' HindIII recognition site
3'...TTCGA▲A...5'

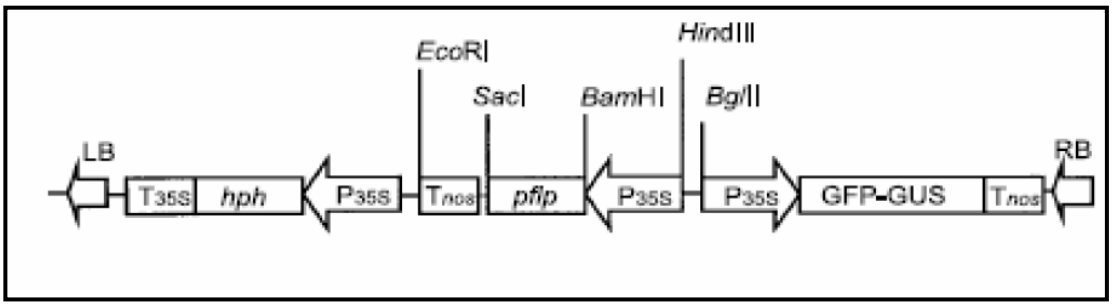

Figure 3.5. T-DNA region of *pflp* plasmid

Agarose gel elecrophoresis results of BamH1 and BamH1/HindIII cut genomic DNAs of wild type tobacco plant for 1,5 and 3 hours (**Figure 3.6**) and for 4, 8 and 16 (overnight) hours reaction times (**Figure 3.7**) and T10-line *pflp*-inserted tobacco plants at the same conditions (**Figure 3.8** and **3.9)** are illustrated, respectively.

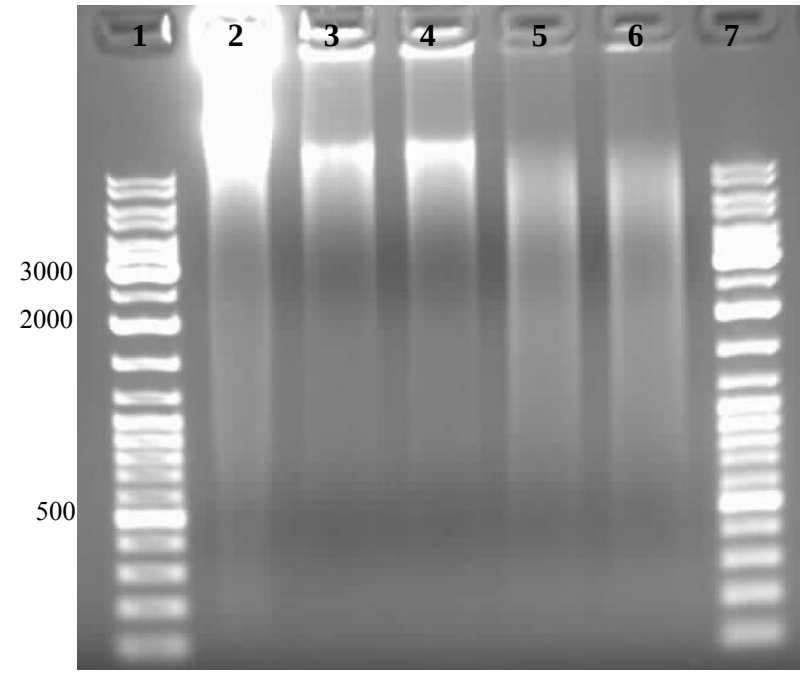

Figure 3.6. 1% Agarose gel electrophoresis of BamH1 and BamH1/HindIII digested wild type tobacco DNAs at two different reaction times. Lane1&7; Lambda/HindIII,2 (MBI Fermentas-#SM0101)(6µL), Lane2; Total 5µg undigested DNA, Lane3&4; Total 5µg BamH1 digested DNAs for 1,5 and 3 hours, respectively, Lane5&6; Total 5µg BamH1/HindIII digested DNAs for 1,5 and 3 hours, respectively.

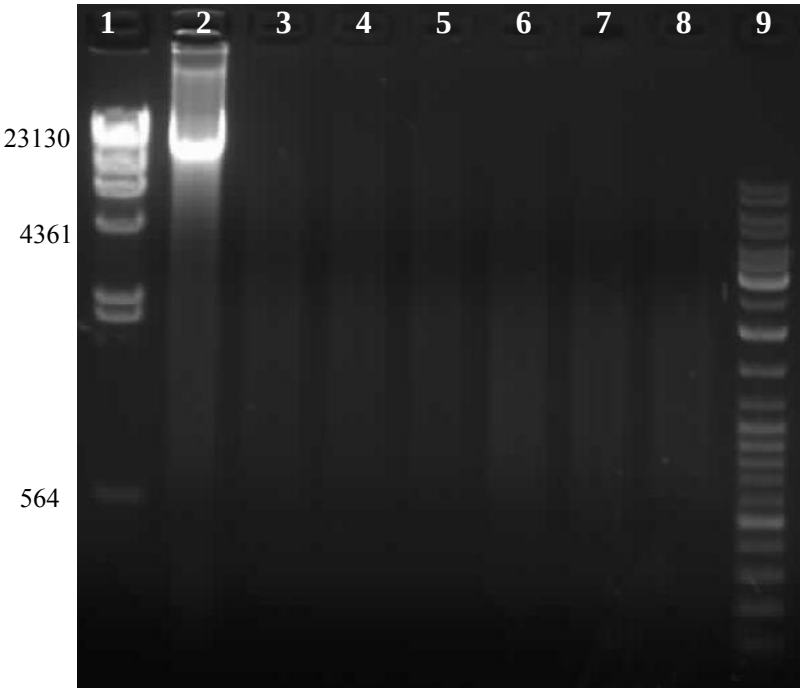

Figure 3.7. 1% Agarose gel electrophoresis of BamH1 and BamH1/HindIII digested wild type tobacco DNAs at three different reaction times. Lane1; Lambda/HindIII,2 (MBI Fermentas-#SM0101)(6µL), Lane2; Total 5µg undigested DNA, Lane3&4&5; Total 5µg BamH1 digested wild type tobacco genomic DNAs for 4, 8 and 16 hours, respectively, Lane6&7&8; Total 5µg BamH1/HindIII digested wild type tobacco genomic DNAs for 4, 8 and 16 hours, respectively, Lane9; Lambda/HindIII,2 (MBI Fermentas-#SM0101)(6µL).

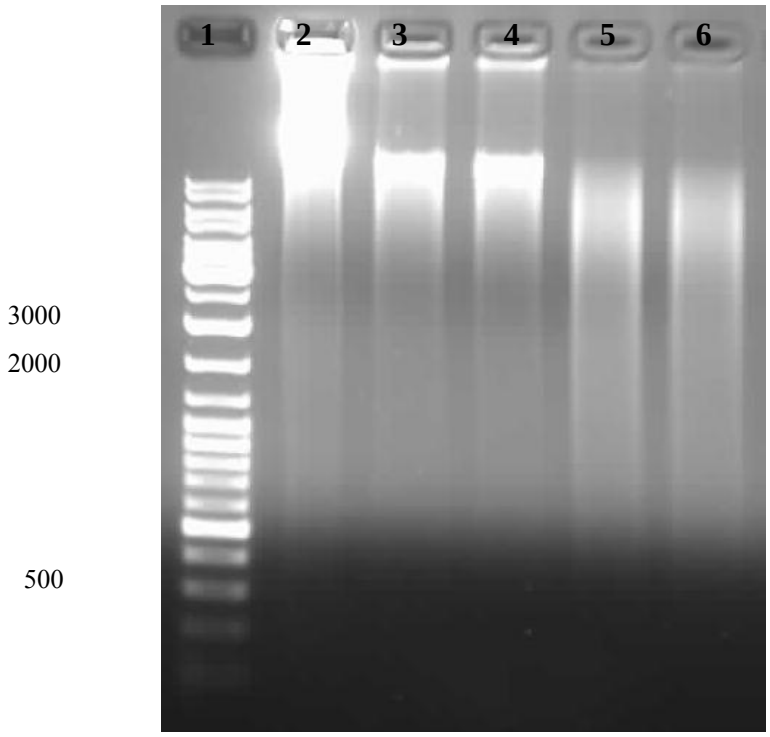

Figure 3.8. 1% Agarose gel electrophoresis of BamH1 and BamH1/HindIII digested T10-line *pflp*-inserted tobacco DNAs at two different reaction times. Lane1&7; Lambda/HindIII,2 (MBI Fermentas-#SM0101)(6µL), Lane2; Total 5µg undigested DNA, Lane3&4; Total 5µg BamH1 digested DNAs for 1,5 and 3 hours, respectively, Lane5&6; Total 5µg BamH1/HindIII digested DNAs for 1,5 and 3 hours, respectively.

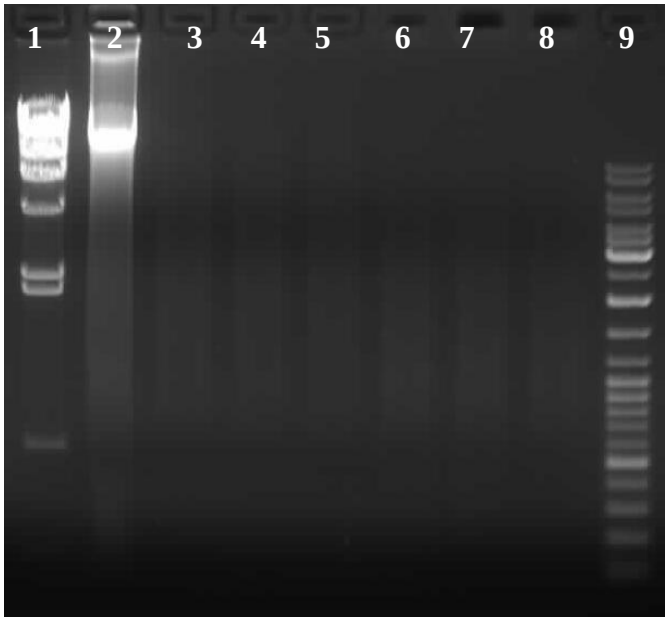

Figure 3.9. 1% Agarose gel electrophoresis of BamH1 and BamH1/HindIII digested T10-line *pflp*-inserted tobacco DNAs at three different reaction times. Lane1; Lambda/HindIII,2 (MBI Fermentas-#SM0101)(6µL), Lane2; Total 5µg undigested DNA, Lane3&4&5; Total 5µg BamH1 digested wild type tobacco genomic DNAs for 4, 8 and 16 hours, respectively, Lane6&7&8; Total 5µg BamH1/HindIII digested wild type tobacco genomic DNAs for 4, 8 and 16 hours, respectively, Lane9; Lambda/HindIII,2 (MBI Fermentas-#SM0101)(6µL).

3.2.2.2. Sonication of Genomic DNAs

5 µg total in 100µL ultrapure water, wild type and T10-line *pflp*-inserted genomic DNA samples were subjected to ultrasonic distruption for 15 seconds time intervals from 0 to 90 seconds in order to get smaller DNA fragments. Agarose gel elecrophoresis was used to determine the size distribution of DNA fragments. Photographs of sonicated wild type and T10-line *pflp*-inserted genomic DNAs are illustrated in **Figure 3.10** and **3.11**, respectively.

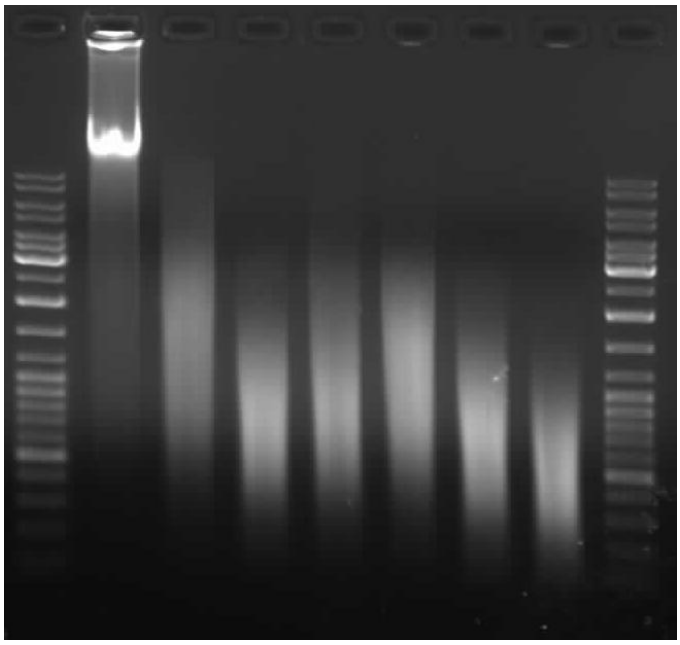

Figure 3.10. Distribution of sonicated wild type tobacco DNA (Total 5µg in each lane) on 1% agarose gel. Lane1&9; Gene Ruler™ DNA ladder mix (MBI Fermentas-#SM0331)(6µL), Lane2; Total 5µg undigested DNA (0sec), Lane3; 15sec, Lane4; 30sec, Lane5; 45sec, Lane6; 60sec, Lane7; 75sec, Lane8; 90sec.

Highly fragmented DNAs are evident from the presence of DNA smears rather than high-molecular weight bands that were eliminated from samples sonicated for 75 or 90 seconds. Longer sonication reduced fragment lenghts to approximately 0.1-1.5kb for both wild type and T10-line *pflp*-inserted tobacco genomic DNAs. P35S promoter region that will be screened by piezoelectric means has a lenght of nearly 735bp and it was expected that fragmented DNA samples contained the significant gene sequence to be surveyed.

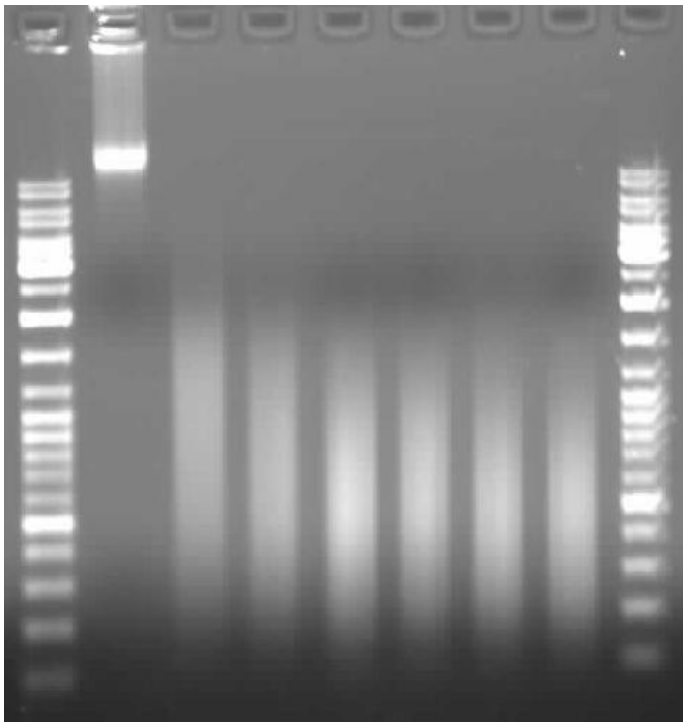

Figure 3.11. Distribution of sonicated T10-line *pflp*-inserted tobacco DNA (Total 5µg in each lane) on 1% agarose gel (1X TAE buffer). Lane1&9; Gene Ruler™ DNA ladder mix (MBI Fermentas-#SM0331)(6µL), Lane2; Total 5µg undigested DNA (0sec), Lane3; 15sec, Lane4; 30sec, Lane5; 45sec, Lane6; 60sec, Lane7; 75sec, Lane8; 90sec.

3.3. GUS Histochemical Analysis of Transgenic Tobacco Plants

β-glucuronidase gene is co - transformed with the *pflp* gene, so we assumed the presence of *pflp* gene by performing histochemical GUS assay to T6 and T10 lines of trangenic tobacco plants. Wild type tobacco plants were used as a control that they do not have this gene naturally in their genomes.

Histochemical GUS assay was performed to test the fate of the transferred *gus* reporter gene with *pflp* gene in T-DNA of the CAMBIA 1304/35S SAPI vector (**Appendix A**). The *gus* gene codes for the β-glucuronidase enzyme which cleaves the substrate 5-bromo-4-chloro-3-β-D glucuronide (X-Gluc) and produces insoluble blue precipitate; dichloro-dibromoindigo. Thus, tissues expressing GUS activity were observed as blue in colour in transgenic lines and no GUS activity was seen in control wild type plants. GUS histochemical assay results were photographed in general and closer views are illustrated in **Figure 3.12** and **Figure 3.13**, respectively.

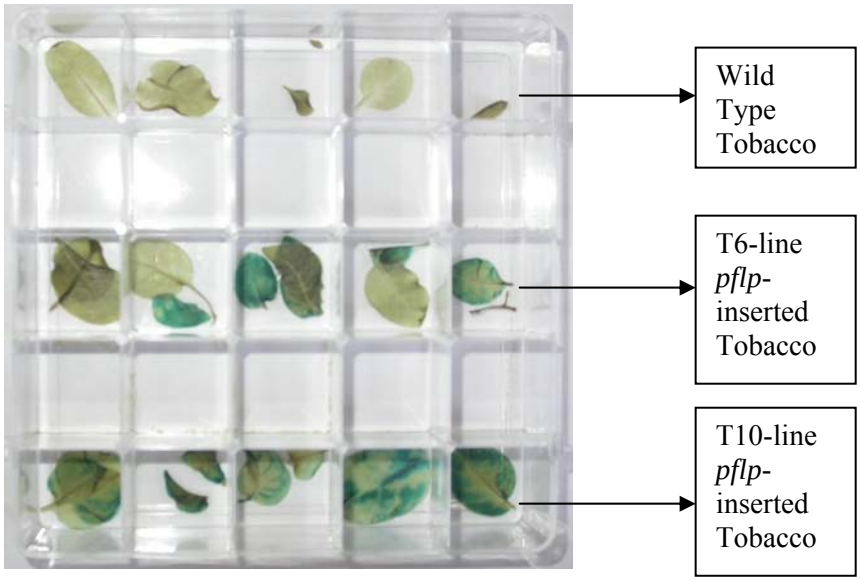

Figure 3.12. GUS histochemical assay results in leaves of wild type tobacco (control), T6-line and T10-line pflp-inserted tobacco plants.

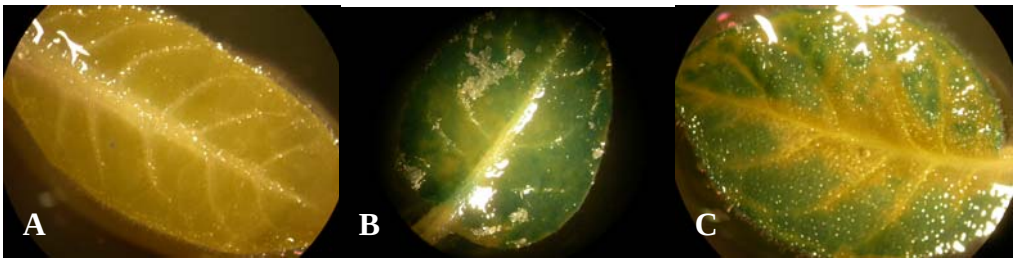

Figure 3.13. GUS histochemical assay results in leaves of (**A**) wild type tobacco (control), (**B**) T6-line pflp-inserted tobacco, (**C**) T10-line pflp-inserted tobacco. (Magnification;10X)

3.4. QCM-based GMO Detection

In this study, the development of a DNA-based piezoelectric biosensor for the detection of CaMV 35S promoter region in genetically modified plants was investigated.

Hybridisation detection is performed following the frequency changes resulting from the interaction between the CaMV 35S specific probe immobilised on the gold electrode of a quartz crystal and the complementary strand in the sample solution.

3.4.1. Probe Immobilisation

QCM system was utilised for *in situ* monitoring and quantification of immobilised probe oligonucleotides (25 mer) on gold (Au) surface either through thiol - Au interaction with thiol - derivatised probe or through amino - aldehyde covalent bonding with amino - derivatised probe. The efficiencies and functionalities of both immobilisation procedures are characterized by hybridisation with 1 µM CaMV 35S target oligonucleotides.

3.4.1.1. Thiol-derivatised probe immobilisation

Immobilisation of thiol-derivatised probe (5' – SH - probe) was achieved by directly interacting the gold surface of the quartz crystal with the thiolated probe solution. The driving force of the coverage of thiolated probes on gold surfaces is due to the formation of strong thiol-gold bonds which has a chemisorption energy of ~30 kcal/mol (Ulman, 1996). Application of the method is easy and straight forward and used commonly by many researchers (Cho *et al.,* 2004; Tombelli *et al.,* 2004; Mannelli *et al.*, 2003(a),(b), 2005). The gold electrode of the QCM sensor provides a transduction surface and the immobilisation of thiolated probes on the gold surface can be monitored in real-time without using a labelling system.

In this study, after successful cleaning of the quartz crystal, thiol-derivatised probe was immobilised either by adding the solution to the cell well of the QCM holder through dipping method or by flowing at a rate of 50 - 100 µL/min through the QCM flow - cell set - up for a period of time. A decrease of 77 ± 4 Hz was observed upon 2 h immobilisation of 2 µM thiolated-probe. Assuming the Sauerbrey equation ($\Delta f = - C_f . \Delta m$) and a sensitivity ($C_f$) of 0.057 Hz (ng / cm^2) for 5 MHz AT - cut quartz crystal at room temperature (SRS, 2004), ~ 1.35 µg / cm^2 mass increase was detected and the surface coverage was calculated to be 1.038 x 10^{14} molecules / cm^2 (MW of thiolated - probe; 7832 g / mole). **Figure 3.14** shows a representative data of a typical time course of frequency change upon the introduction of 2 µM thiolated-probe in 1 M KH_2PO_4; pH:3.8 (immobilisation solution) to the QCM flow - cell for an overnight time period (16 h).

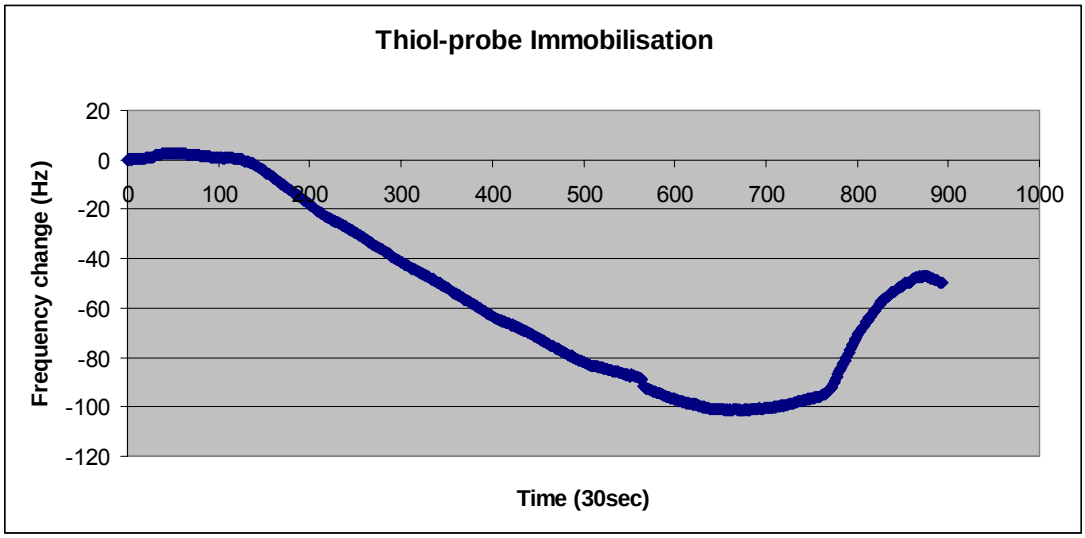

Figure 3.14. QCM frequency change versus time for the immobilisation of 2 µM (15.6 ng/µL) thiol-derivatised probe in a QCM flow-cell for an overnight time period.

Following immobilisation with thiolated - probe, the QCM surface was treated with a blocking thiol; 1 mM of 6 - mercapto 1 - hexanol (MCH ; $HS(CH_2)_6OH$) for 1 h, in order to reduce the non - specific and weakly - bonded adsorption of oligonucleotides on the gold surface. An additional adsorption of mercaptohexanol gave a frequency drop as much as 217 ± 6 Hz, which corresponds to an additional adsorbed mass of ~ 2.456 µg / cm^2 and the surface coverage was calculated to be 1.1 x 10^{16} molecules / cm^2 (MW of MCH; 134.24 g / mole). **Figure 3.15** shows a representative data for the frequency decrease responding to the addition of blocking thiol solution for 1 h.

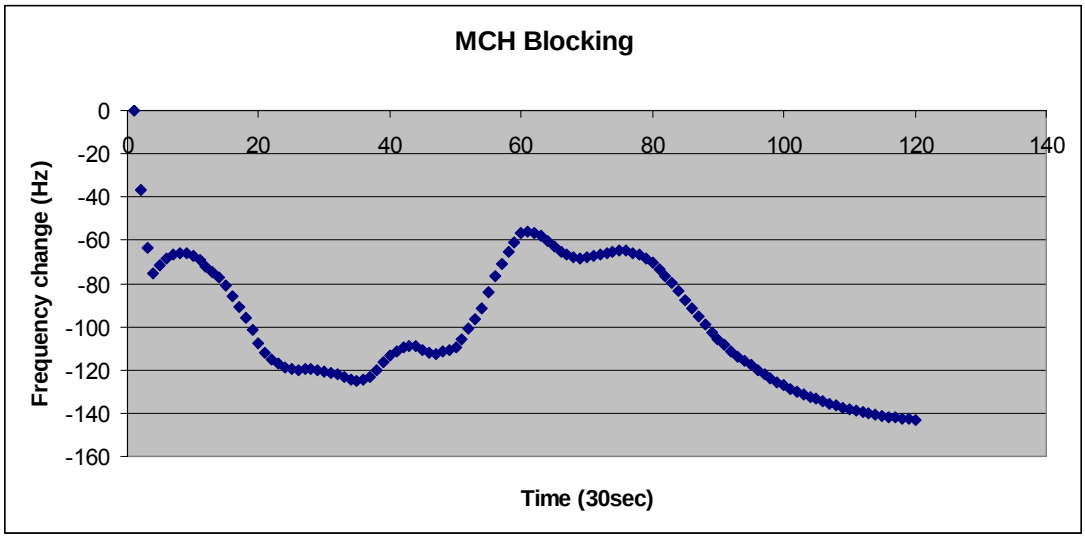

Figure 3.15. QCM frequency change (Hz) versus time (30 sec) for the application of 1mM 6-mercapto-1-hexanol (MCH) blocking thiol solution for 1h in order to block the non-specific binding sites on the gold sensor surface.

3.4.1.2. Amino-derivatised probe immobilisation

Amino-derivatised probe oligonucleotides were immobilised covalently to the gold surface of the QCM by using the plasma polymerised ethylenediamine (EDA) as an interface. EDA polymerised film surface was functionalised by activating the amino groups with gluteraldehyde to form aldehyde groups flanking in the immobilisation solution to make covalent bonds with amino-derivatised probe oligonucleotides.

3.4.1.2.1. Surface Modification of Quartz Crystals

3.4.1.2.1.1. Glow-discharge treatment

Glow-discharge treatment is commonly used to change the surface characteristics of materials in many applications (Muguruma and Karube, 1999; Duman *et al.,* 2003; Bouaidat *et al.,* 2005; Akdoğan *et al.,* 2006). Studies indicated that ethylenediamine plasma-polymerised films formed on the gold electrodes of the quartz crystals were extremely thin and homogenous. Also, in the study of Muguruma and Karube, 1999, it was shown that the QCMs covered with plasma-polymerized ethylenediamine oscillated in a stable manner and had a lower noise level in 0.1 M PBS (Phosphate-buffered saline) when compared with other conventional matrix coatings namely, polyethyleneimine (PEI) and (δ-aminopropyl) trimethoxysilane (APTES). Moreover, there are large amounts of amino groups which enable the immobilisation of antibodies or functionalised oligonucleotides.

In this study, ethylenediamine was chosen as an active monomer in plasma polymerisation in order to create amino-like active groups on the gold electrodes of quartz crystals for the immobilisation of amino-derivatised P35S probe. Conditions for plasma polymerisation were set as, the discharge power: 20Watt; treatment time: 5 minutes; and the monomer flow rate: 35 ml/min. The base frequencies of every QCM crystals were determined before and after the plasma modification with the frequency counter. A significant decrease in the base frequencies were observed due to the EDA monomer deposition on the crystal surfaces. Upon glow-discharge treatment, the average frequency shift and standard deviation of these crystals in

immobilisation solution was 1594 ± 18 Hz. The thickness of the EDA film created on quartz crystal was calculated from the equation; ($T_f = \Delta m / \rho_f$), where, T_f is the thickness of the polymeric film on the crystal surface (cm); Δm, the change in mass per unit area in g / cm^3 (calculated from Sauerbrey's equation); and ρ_f is the density of the film material; herein, EDA monomer (0.895 g / cm^3). The thickness of the resulting EDA polymer on quartz crystal was calculated to be 26 ± 7 Å. The thickness value obtained at the set conditions of plasma polymerisation in this study is compared with the value obtained in a previous study of Duman *et al.,* 2003, in which the glow-discharge conditions for EDA plasma-polymerisation were 15 Watts; 2.5 minutes; 30 ml / min and yielded a thin film of 43 ± 24 Å thickness. According to findings in the study of Saber (2001) it was found that as discharge power increased, frequency shifts decreased meaning that the deposition on the quartz crystal decreased. In the same study it was noted that the surface modifications achieved at lower discharge powers (especially at 5 Watts) were not stable and desorption of the polymerised layer was observed after subsequent washing with water and that result was explained by the findings of Yasuda (1985), as the accumulation of waxy layer on the surface at lower applied powers. Moreover, less accumulation of mass and a color change of the gold electrodes were observed at high discharge powers (especially 30 Watts) due to etching of the layer even at the electrode material (Saber, 2003). In this study, a 5 Watt-increase in discharge power and doubled exposure time might caused the difference; nearly half, in thickness of the deposited ethylenediamine layer on the quartz crystals surfaces, but we also observed that the values correlate with each other when the standard deviations are taken into account.

3.4.1.2.1.2. Gluteraldehyde Attachment

After plasma polymerisation of ethylenediamine on quartz crystal surfaces, chemical activation of the surface was achieved by using gluteraldehyde (GA) as a crosslinker. GA is the most commonly used agent for crosslinking of amine groups in compounds like proteins or oligonucleotides (Hermanson, 1996; Duman *et al.,* 2003). The plasma polymerised crystals were immersed in GA solution (2.5 % w/w in phosphate buffer, pH:7.0) for 16 hours at room temperature. The gluteraldehyde application conditions were previously studied and optimised (Saber *et al.,* 2002; Duman *et al.,*

2003). Application time of 16 h (overnight) was chosen for practical purposes since no significant increase was found between 2 h and 16 h (Duman *et al.*, 2003). The frequency changes of the crystals were monitored by the QCM system during the gluteraldehyde activation. Since GA is a dialdehyde, it is expected that one aldehyde group covalently interacts with an amine group on EDA, while the other aldehyde group is free for immobilisation with amino-derivatised P35S probe oligonucleotide. Proceeding reactions in EDA plasma polymerisation and gluteraldehyde chemical treatment steps are shown in **Figure 3.16**.

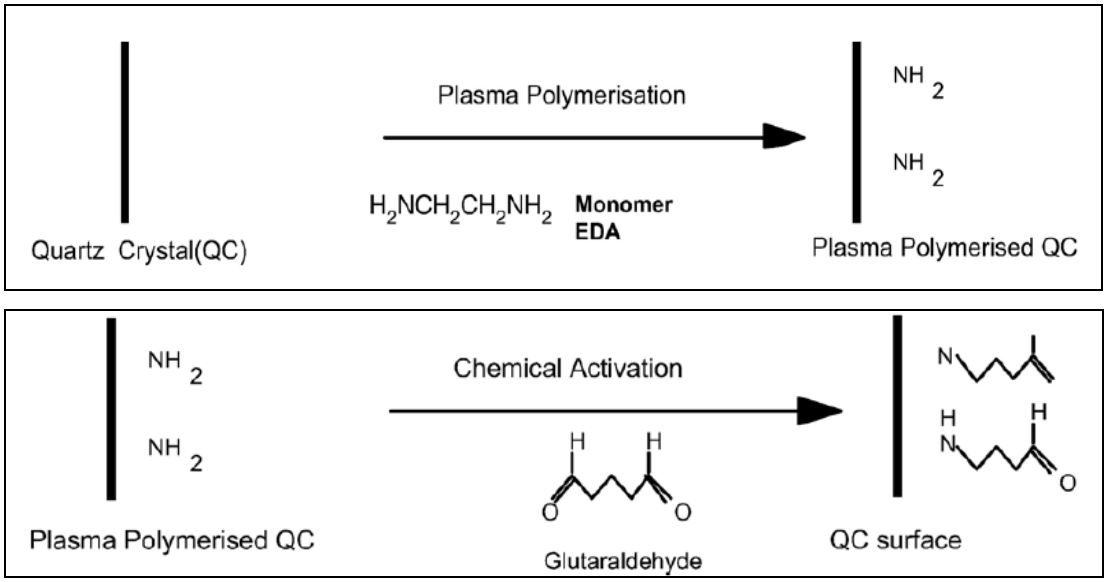

Figure 3.16. Reaction steps in plasma polymerisation and gluteraldehyde activation (Akdoğan *et al.*, 2006).

A representative data of a frequency change during gluteraldehyde activation of an EDA plasma-polmerised quartz crystal for an overnight time period is shown in **Figure 3.17**. From the measurements of five independent plasma-polymerised ethylenediamine covered QCMs, an average of 74 ± 18 Hz frequency decrease was calculated for the gluteraldehyde interaction. The frequency measurements was taken when the QCM holder was soaked in the gluteraldehyde solution which was stirred at a 200 rpm rate on a magnetic stirrer in the dark at room temperature. Stirring is required in order to reduce excess gluteraldehyde accumulation on the ethylenediamine polymer surface. As a preliminary result, as high as 855 Hz of frequency decrease was observed for a polymerised crystal in GA activation without

stirring. Although immobilisation of amino-derivatised probe was detected on the surface of that QCM, it did not produce reproducible hybridisation results which meant that there was still removal of excess gluteraldehyde from the QCM surface.

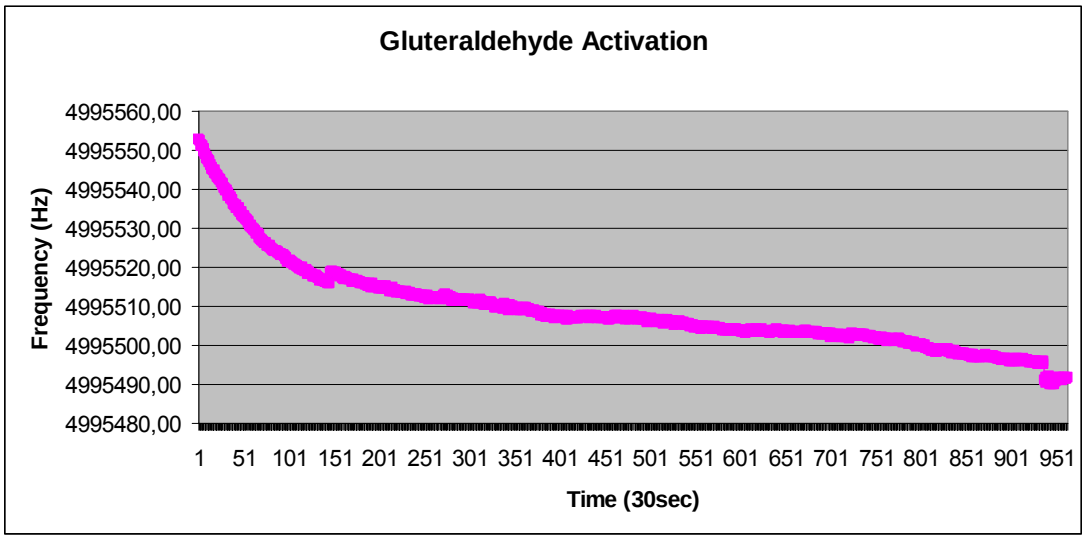

Figure 3.17. Frequency change versus time data of a gluteraldehyde activation of ethylenediamine plasma polymerised QCM.

3.4.1.2.1.3. Amino-probe Immobilisation

After surface functionalisation of the quartz crystals with gluteraldehyde, amino-derivatised probe immobilisation was performed by adding 500 µL of 2 µM (15.6 ng / µL; same concentration with thiolated-probe) probe solution into the QCM cell well, for 2 h. Frequency changes were measured in immobilisation solution (1M KH_2PO_4; pH:3.8) and basal frequencies before and after probe immobilisation were recorded. An average of 78 ± 43 Hz frequency decrease was observed upon amino-probe immobilisation of five independent surface functionalised (one free aldehyde groups from gluteraldehyde) quartz crystals. According to Sauerbrey's equation, the mass increase on the surface is ~ 1.37 µg / cm^2 and the calculated surface coverage is 1.054 x 10^{14} molecules / cm^2 (MW of amino-derivatised probe; 7815.2 g / mole). A representative data of frequency decrease in QCM oscillation upon immobilisation with amino - derivatised probe is shown in **Figure 3.18**.

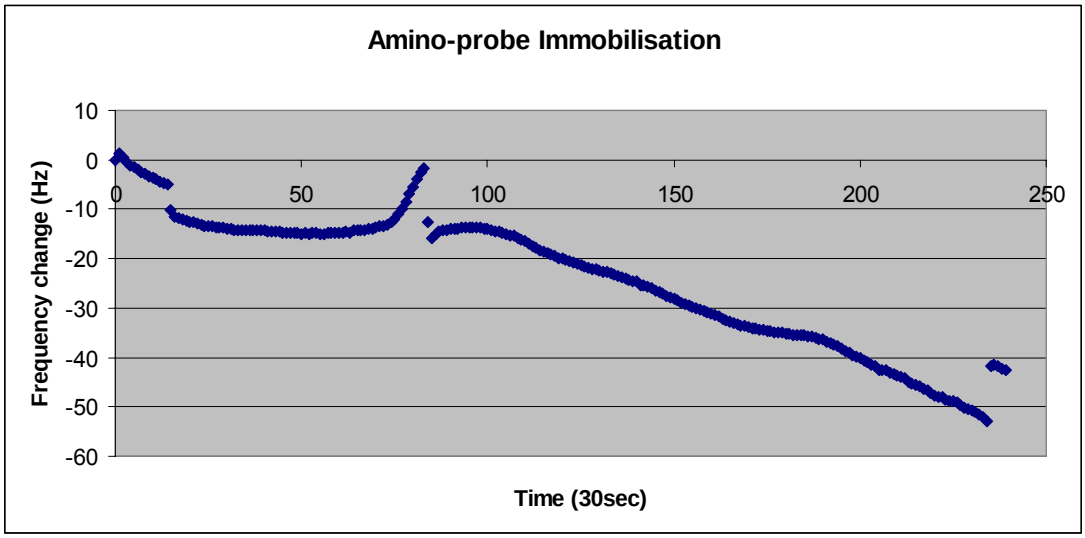

Figure 3.18. QCM frequency change versus time for the immobilisation of 2 µM (15.6 ng/µL) amino-derivatised probe in a QCM flow-cell for a 2h time period.

3.4.2. Analytical Performances of Immobilised Probes

The efficiencies of both thiolated and amino-derivatised probe immobilisation procedures were further evaluated by comparing the obtained frequency values, mass values deposited on the quartz crystal surface and calculated surface coverages for a constant 2 µM probe concentration and 2h immobilisation time. The values obtained from the immobilisation procedures are summarised in **Table 3.1**.

Table 3.1. Frequency changes, mass deposition and surface coverage values upon immobilisation procedures.

Probe immobilised	$\Delta F_{immobilisation}$ (Hz)	$\Delta F_{MCH\text{-}blocking}$ [a] $\Delta F_{GA\ activation}$ [b]	Δm (µg/cm^2)	Surface coverage (molecules/cm^2)
Thiol	(-)77 ± 4	(-)217±6 [a]	1.35	1.038 x 10^{14}
Amino	(-)78± 43	(-)74±18 [b]	1.37	1.054 x 10^{14}

For the same probe concentration (2 µM= ~ 15.6 ng / µL) and immobilisation time (2 h), both probe types showed the same immobilisation pattern. But it should be noted that the frequency decrease of thiolated - probe was observed before MCH blocking while the frequency decrease of amino - probe was observed after gluteraldehyde activation.

So, the additional thiol - blocking step with MCH might changed the surface characteristics of the thiolated - probe immobilised crystals since it was expected that mercaptohexanol would fill up the pinholes and collapse sites (**Figure 3.19 (a,b)**) on the thiol monolayer and exchange with the non - specific bonds and eliminate weak or non - oriented (**Figure 3.19 (c)**) thiol - probes on the gold surface of the QCM.

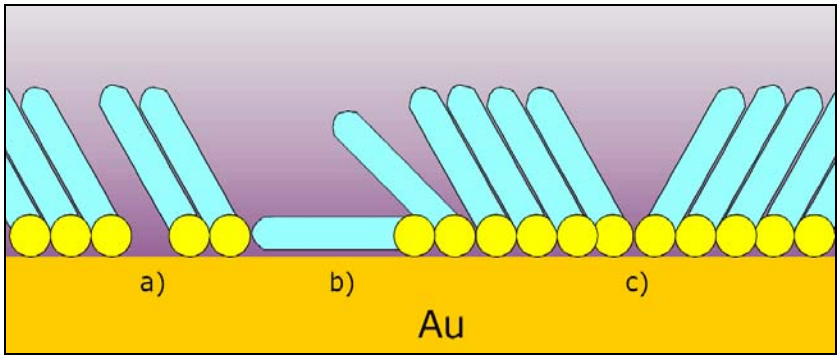

Figure 3.19. Possible defects of the thiol monolayer. a) pinholes, b) collapse sites, c) boundries of different oriented domains (Wrobel, 2001).

For better evaluation of the efficiencies of both immobilisation methods, hybridisation characteristics with the P35S target probe was investigated. Frequency changes were measured after hybridisations with 0.5 µM (3.85 ng / µL), 1µM (7.7 ng / µL) and 2 µM (15.4 ng / µL) target concentrations for thiolated and amino-probe immobilised QCMs. The frequency decreases upon hybridisation with changing target concentrations is shown in **Figure 3.20**. Slightly higher frequency decreases were observed in hybridisation processes with QCMs immobilised with the thiolated-probe. Also, it is worth to mention that, more than 5 readings could be detected for hybridisation of thiolated - probe and target oligonucleotides after consecutive regeneration of the system with 1 mM HCl, but barely 3 readings for amino-probes, which means reproducibility of the thiol - probe immobilised QCMs was better than the amino ones. That failure of reproducibility in hybridisation detection in amino - derivatised probe immobilised QCMs can be explained by the elimination of the ethylenediamine polymer layer from the gold surface since the base frequency level before the immobilisation steps was reached quickly.

Although plasma polymerisation is a promising method for the covalent modification of amino - derivatised probe oligonucleotides to the electrode surface of the QCM sensor, it needs further investigation in the optimisation of discharge conditions and subsequent immobilisation and hybridisation processes. Hence, thiol - probe immobilisation method was chosen for the further experiments in developing a QCM - based DNA sensor for the P35S sequence.

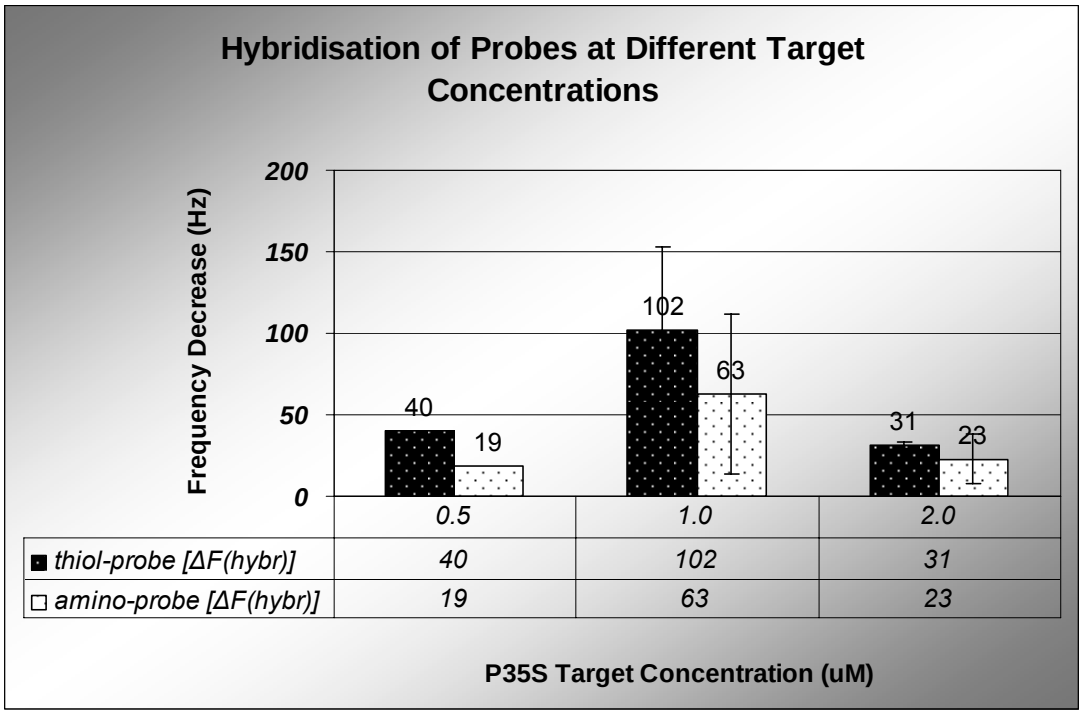

Figure 3.20. Frequency changes on both thiol and amino-derivatised P35S probe immobilised QCMs upon hybridisations with changing concentrations of target oligonucleotides. (1 µM target oligonucleotide = 7.7 ng / µL). Standard deviations are given as bars.

3.4.3. Calibration of the QCM sensor with Synthetic Thiol-Oligonucleotides

The calibration of the thiol modified QCM sensor was achieved by hybridisation experiments in the flow - cell set - up of QCM apparatus, with different concentrations of synthetic P35S target oligonucleotides. The thiol probe immobilisation was done with 2 µM thiolated P35S probe in immobilisation solution for 2 h, following a blocking step with 1 mM mercaptohexanol (MCH) for 1 h. The frequency change was recorded by measuring base frequencies in hybridisation

buffer before and after the hybridisation reaction. **Figure 3.21** shows the calibration curve of the thiol - modified crystal.

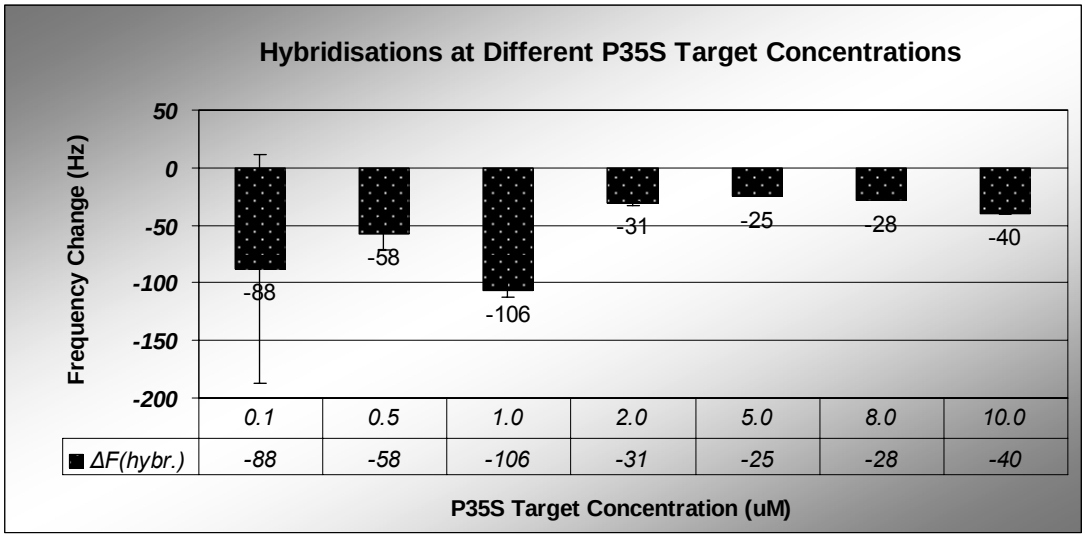

Figure 3.21. Calibration curve obtained from synthetic P35S target oligonucleotides on the crystal modified with 2 μM (15.6 ng / μL) thiolated probe for 2 h. The error bars represent the standard deviations.

The graph indicates that hybridisation of synthetic target oligonucleotides was somewhat inhibited when an excess amount of oligonucleotide complementary to the probe was present in the buffer solution. This can be due to the increased stearic hindrance at high concentrations of the target oligonucleotide resulting in the formation of nonspecifically binded double - strands in the solution by the two single strands rather than the hybridisation with the probe oligonucleotide immobilised on the QCM surface.

In other studies reported in the literature, calibration of the sensors was done with synthetic target (25mer) concentrations lower than 1.0 μM (Tombelli *et al.,* 2005; Su *et al.,* 2005). The calibration curves obtained with the same target oligonucleotides for 35S probe showed a profile with a linear region between 0 and 0.1 μM followed by a plateau (Minunni *et al.,* 2001a,b; Mannelli *et al.,* 2003, 2005). An experimental detection limit of 10nM was obtained in the study of Mannelli and co-workers (Mannelli *et al.,* 2005). Considering the results of other studies, the highest frequency decrease at 1 μM target concentration which was obtained in our study,

was found probably at the saturation level, in the plateau region (**Figure 3.21**). Being at the saturation level, 1μM synthetic target oligonucleotide concentration was chosen for the hybridisation reactions in the optimisation of the thiol-modified probe immobilisation experiments.

3.4.4. Optimisation of the Thiol-probe Immobilised QCM Sensor

The probe concentration, immobilisation time and presence of blocking agent; mercaptohexanol (MCH) were the parameters investigated for the optimisation of the thiol-probe immobilisation of the QCM sensor. The optimised immobilisation conditions were further used for the detection of P35S sequence in PCR amplified and genomic DNA samples.

3.4.4.1. Thiol-probe Concentration

Optimisation of thiol-probe concentration was investigated by immobilising different probe concentrations (0.5-10.0 μM) on different QCM surfaces following 1h MCH blocking or not. Frequency decreases were detected from the measurements of base frequencies in immobilisation solution before and after the immobilisation processes. **Figure 3.22** illustrates the profile of frequency decrease upon immobilisation with changing concentrations of thiol-probe. Furthermore, the efficiencies of immobilised thiol-probes in hybridisation process were tested by hybridising with 1μM target concentration. Frequency changes were undetectable from 0.5 and 1.0 μM thiol-probe immobilised QCMs so they were not included in the graph of hybridisation results. **Figure 3.23** shows the frequency decreases of QCMs immobilised with changing concentrations of thiol-probe upon hybridisations with the target oligonucleotide. Both immobilisation and hybridisation results were obtained from two independent sets of experiments.

QCM sensors immobilised with 2μM and 5 μM thiol-probes resulted in the highest surface coverages of the probe, due to the highest frequency decreases after the immobilisation process with or without MCH blocking. Consequently, hybridisation results of those QCMs with the target oligonucleotide were in accordance with the

immobilisation results, except the hybridisation frequency decrease obtained from the 5 μM thiol-probe immobilised QCM without blocking agent. The possibility of obtaining that abnormal amount of frequency decrease will be explained in the proceeding subtitle.

A 2 μM thiol-probe concentration was determined as the optimal value for further immobilisation processes since utilising lower probe concentrations would be suitable when practical and economical concerns are also taken into account.

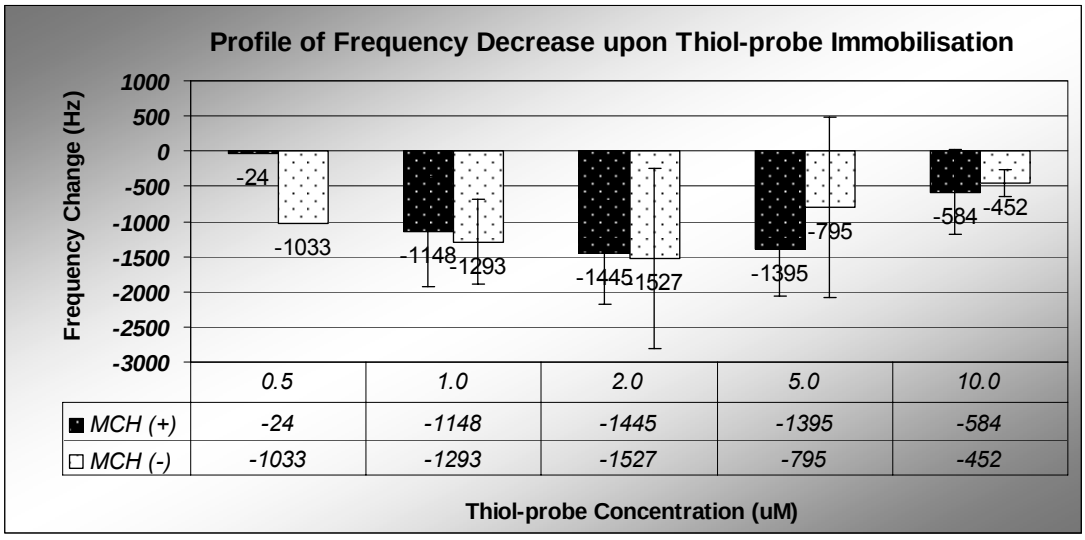

Figure 3.22. Frequency decrease upon immobilisation of the quartz crystals with changing concentrations of P35S thiol probe oligonucleotide with or without MCH (1mM in ultrapure water for 1h) blocking. (1μM thiol-probe oligonucleotide = 7.8 ng/μL). The error bars represent the standard deviations.

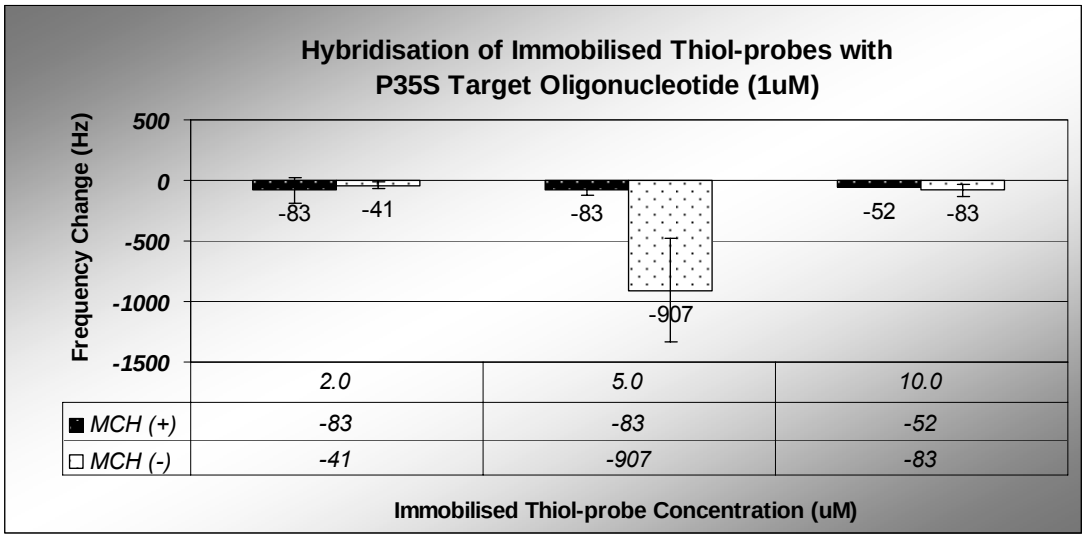

Figure 3.23. Frequency decreases of QCMs immobilised with changing concentrations of thiol-probe upon hybridisations with 1µM P35S synthetic target oligonucleotide. The error bars represent the standard deviations.

3.4.4.2. Presence of Blocking Agent (MCH)

The probe concentration determination was investigated with the presence or absence of blocking agent mercaptohexanol (MCH). The efficiency of immobilisation procedure was evaluated according to the results of the frequency changes after the hybridisation with the target oligonucleotide. The results were explained in the previous title **3.5.4.1.** Herein, the presence of blocking agent MCH will be evaluated for the efficiency of the QCM sensor immobilised with thiol-derivatised probe.

The graph in **Figure 3.**22, representing the frequency decreases upon immobilisation procedures, close amounts of immobilisation frequency decreases were observed for the QCMs either blocked with mercaptohexanol or not. But, standard deviations obtained from the frequencies of QCMs that were not blocked by mercaptohexanol (MCH(-)) were so high. Treatment with the blocking agent provided reasonable hybridisation results with the target oligonucleotide. That could be due to the fact that the probe oligonucleotide was made to 'stand up' on the gold surface due to conformational crowding by the MCH, giving access to complementary strands. The abnormal frequency change obtained from the hybridisations on the 5µM thiol-probe immobilised QCM without MCH blocking had a high standard deviation (**Figure**

3.23). That abnormality in frequency change could resulted from the inconsistencies during the measurement processes. Mostly, fluctuations in voltage values during the experimentation resulted in extereme frequency changes that misleaded in interpretation of the the mass loaded.

One of the observed results during the immobilisation experiments was the slight increases in the frequency values of the thiolated-probe immobilised QCMs upon the adsorption of MCH on the surface. That result can be explained by the desorption of the probe from the gold surface. Steel and co-workers interpreted that the probe desorption during the MCH treatment step might be a result of thiol-thiol exchange, rather than thiol displacement of nucleotide-gold contacts (Steel *et al.,* 2000). MCH and probe exchange then resulted in the mass changes on the QCM surface since MCH has a lower molecular weight (134.24 g/mol) when compared with the P35S thiol-probe (~7832 g/mol) immobilised on the QCM surface. It acts as a diluent by forming mono self assembled layers on the gold QCM surface through its thiol group. From the same study of Steel *et al.,* 2000, it was concluded that, the desorption was also aided by the electrostatic repulsions between adsorbed probes at high initial surface densities but the displacing action of MCH was the dominant factor in determining the final surface coverage.

The experimental sheme is represented in **Figure 3.24** illustrating the initial immobilisation of single stranded thiolated-P35S probe oligonucleotide on the gold surface of the QCM, followed by MCH adsorption for blocking the unbounded surface to reduce nonspecific adsorption, and then the hybridisation of the single strand P35S target oligonuclotide with its complementary strand.

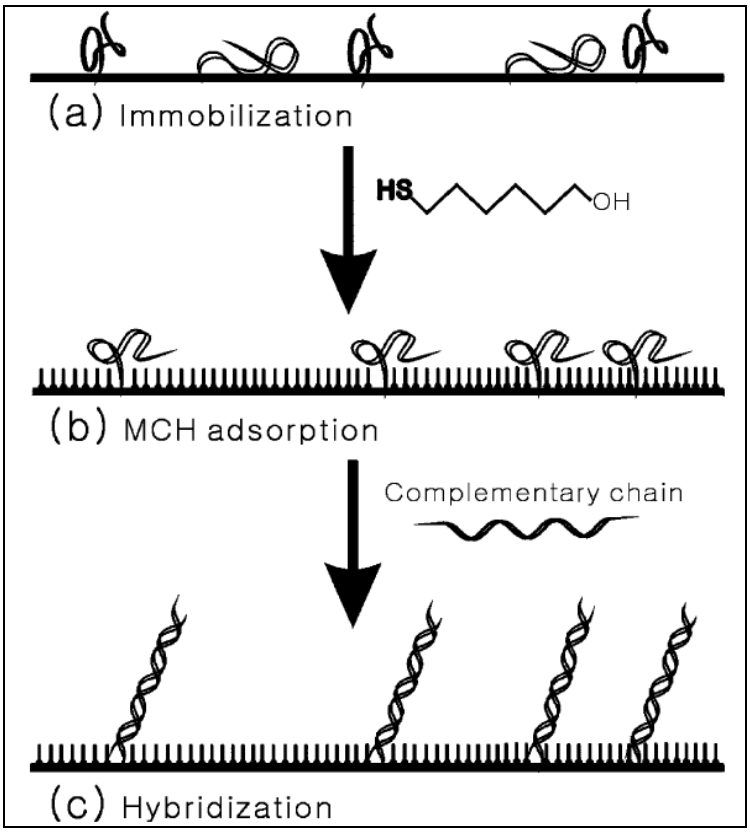

Figure 3.24. Shematic illustration of (a) the immobilisation of DNA strand, (b) subsequent MCH adsorption, and, c) finally hybridisation of DNA strand on the gold electrode surface **(**Ha *et al.,* 2004).

3.4.4.3. Probe Immobilisation Time

In order to observe the effects of probe exposure time on the immobilisation efficiency, the QCMs were incubated with the 2μM thiol-derivatised probes *without* blocking thiol solution (MCH) in immobilisation solution at different periods of time (2, 8 and 16 h). Immobilisation was done in batch-mode at 4°C in order to reduce the DNAse activity. MCH treatment was eliminated from the experiment in order to reduce the time of the experiment.

The efficiencies of immobilised QCMs were evaluated according to the hybridisation results with the target oligonucleotide (1 μM). **Figure 3.25** shows the decreases in frequency values obtained from both immobilisation and consequently, hybridisation processes. The results were obtained from three independent experiments.

The surface coverage of the thiolated-probe increased as the exposure time increased from 2 h to 8h. The higher mass deposited on the surface after 8h immobilisation was characterized by the higher frequency change (ΔF= -225 Hz) upon immobilisation. Lower surface coverage for 16h immobilisation than 8h can be explained by the desorption of probes due to electrostatic repulsions as well as the degredation of the DNA probes by the DNAse activity as the immobilisation time was prolonged.

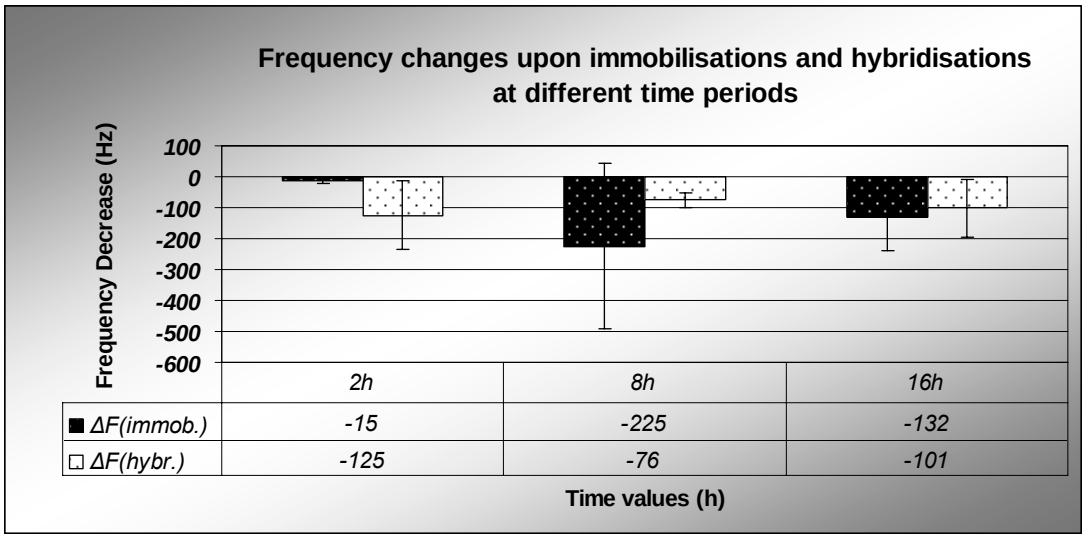

Figure 3.25. Frequency decreases obtained after immobilisations at 2, 8 and 16h durations and related frequency changes after hybridisations with 1μM target oligonucleotide.

QCMs immobilised for 2 hours had the maximum frequency change values after hybridisations with the target oligonucleotide. This can be due to easy access of the target oligonucleotide to its complementary strand immobilised on gold surface since 2 h immobilisation of the thiolated probe gave the minimum freqeuncy decrease indicating less dense surface coverage on the QCM. However, elimination of the blocking and diluent agent mercaptohexanol from the procedure also must be taken into account. Non-specific adsorptions in the absence of blocking agent might also led to positive false results for the hybridisations.

According to the obtained results, the probe concentration of 2 μM and a contact time of 2 h following a 1 h MCH blocking, were adopted for thiol-probe immobilisation conditions and used in the analysis of P35S region PCR amplified, digested and sonicated samples of tobacco genomic DNAs.

3.4.5. Hybridisations with CaMV 35S PCR Products

P35S promoter regions were amplified from the plasmid pTJK 136 by PCR. The amplified 195 bp region was containing the target base sequence complementary to the immobilised thiolated - P35S - probe sequence on the QCMs (**Appendix A**). The collected PCR samples were phenol purified and resuspended in Tris - EDTA (pH:8.0) buffer. The concentration of the stock PCR sample was detected spectrophotometrically and was calculated to be 280 µg / µL.

The PCR amplified DNA samples contained a high number of copies of the target sequence so, hybridisation detections on the QCM sensor was performed with the diluted samples in hybridisation buffer from 1/50 to 1/2000 giving a concentration range of 11.4 to 0.4 µg / µL. The frequency changes upon hybridisations with the diluted samples of PCR-amplified P35S products are given in **Figure 3.26**. The highest frequency change was obtained with the 1/500 diluted PCR sample having a DNA concentration of 1.4 µg / µL and the lowest with the 1/2000 one (0.4 µg / µL DNA concentration). A decreasing trend in frequency changes was observed as the concentration of the sample was increased. That result was expected as confirmed by earlier findings with the target oligonucleotides since the presence of increased number of target sequences in the hybridisation solution caused a stearic hindrance and that hindrance prevented access to the immobilised probe sequences on the QCM surface. Also note that the PCR-amplified samples were double stranded and heat denatured. It can be also suggested that when the concentration of the DNA in the sample increased, the efficiency of denaturation might be reduced and that had led to the regeneration of the strands within the amplified products rather than the immobilised probe on the QCM surface. In some studies, that problem was overcome by treating the PCR samples with blocking oligonucleotides; primers designed for sequences outside the target region and obtained successful results (Mannelli *et al.,* 2005; Tombelli *et al.,* 2006).

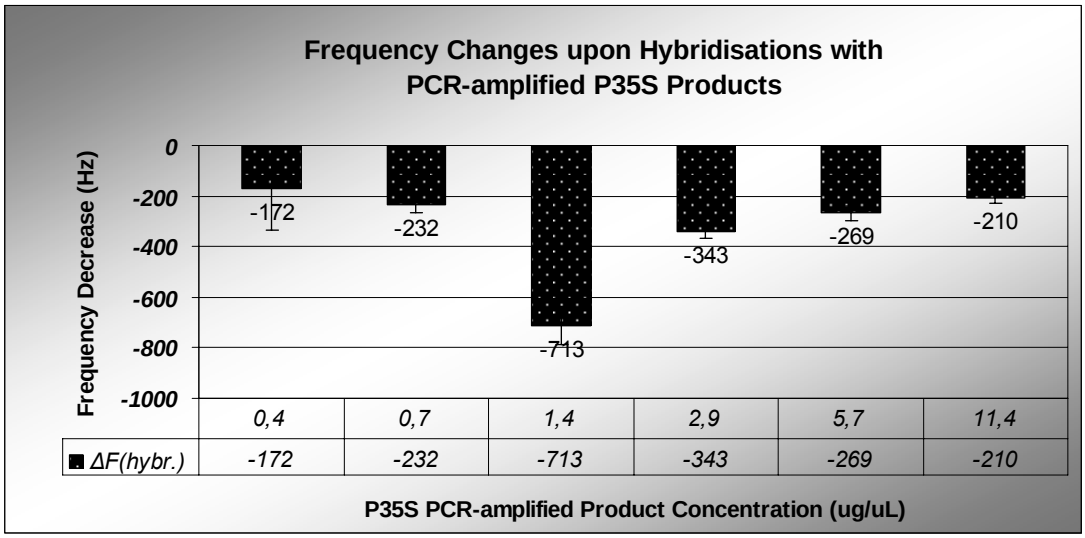

Figure 3.26. Frequency changes on thiol-probe modified QCM sensors upon hybridisations with the PCR-amplified P35S products.

Higher frequency change values were obtained with the PCR - amplified samples (195 bp) when compared with the results of synthetic target oligonucleotides (25 bases). That could be explained by the high mass deposition on the QCM surface resulting from the difference in lenghts of the target sequences. PCR-amplified sample had nearly 8 times longer sequence than synthetic oligonucleotide.

In order to investigate the minimum level of detection, dilutions of PCR products more than 2000 times is needed. The lowest tested concentration of PCR-amplified DNA was 400 ng / µL and it gave a frequency change of (- 172 ± 163 Hz) with a high standard deviation. Minunni *et al.*, (2001) detected highest response from the 480 times diluted samples of 195 bp P35S PCR - amplified products from 2 % transgenic soya flour Certified Reference Material (CRM) when hybridised with the same probe sequence that was used in our study, but on an electrochemical sensor in which the signal was amplified with daunomycin; an intercalating agent used as a hybridisation reaction marker. In most of the studies, frequency changes were reported according to the diluted samples of the PCR - amplified products rather than concentrations which prevented direct comparison of detection limits of the immobilised probe system (Minunni *et al.*, 2001; Mannelli *et al.*, 2003).

In order to check the specificity of the system, 1/200 dilution of a non-complementary PCR product (TNos PCR-amplified DNA; 180 bp) was also tested by hybridising with the thiol-probes immobilised. Non-detectable or minute frequency decreases was observed from independent hybridisation experiments. Obtained minute frequency decreases (< 3 Hz) might mostly resulted from non-specific adsorption of the PCR product.

3.4.6. Hybridisations with Genomic DNA Samples

The major challenge of developing a QCM - based GMO detection is to optimise the system so that the transgenes from genomic DNAs of real samples are detectable without use of the PCR amplification step.

In our study, in order to test the detection ability of the QCM - sensors immobilised with the thiol - probe specific to P35S sequence, digested and sonicated genomic DNA samples from T10 - Line of *pflp* gene - inserted and wild type tobacco plants were used in hybridisation experiments. The T - DNA of the inserted gene included more than one copy of P35S promoter regions (**Appendix D**). Genomic DNAs of wild type tobacco plants were used as a negative control in hybridisation studies since P35S sequence was not found naturally in their genome. Transgenicity of the *pflp* gene - inserted tobacco samples were previously verified with the Roche Applied Science's LightCycler® GMO Screenin Kit, which is a PCR kit for the qualitative detection of genetically modified plants using the LightCycler® System (Tuncer, 2006). The DNAs of wild type and T10 - Line of *pflp* gene - inserted tobacco plants were isolated by using the CTAB method explained in **2.2.3.1**.

3.4.6.1. Digested DNAs

In the first group of experiments, the DNAs that digested with the two restriction enzymes (BamH1 and HindIII) for an overnight time (16 h) were used in hybridisation detection on the QCMs immobilised with the thiolated P35S probe. Digestion with the both enzymes, was expected to free the P35S gene (~ 735 bp) in the reaction medium without interfering the transgene. A total of 5 μg DNA was

digested in a 100 µL total reaction mixture resulting a 50 ng / µL total DNA concentration in the stock sample. Fragmented genomic DNA samples were diluted 50, 100 and 200 times with the hybridisation buffer and 1.0 ng / µL, 0.5 ng / µL and 0.25 ng / µL of final DNA concentrations were obtained, consecutively.

Heat denatured samples of diluted genomic DNAs were tested with the QCMs immobilised with P35S probes, through hybridisation reaction. Heat denaturation step was necessary for genomic DNAs, as they were double stranded. The frequency changes observed from at least three independent measurements with the diluted DNA samples of wild type and transgenic tobacco plants were presented in the graph in **Figure 3.27**. The system was able to detect the complementary P35S target sequences in T10 - Line *pflp* gene - inserted tobacco samples even at a DNA concentration of 0.25 ng / µL (- 6 Hz ± 3). A linear relationship was observed between the tested concentrations of the transgenic DNA samples and the frequency changes observed. It indicated that the DNA concentrations were below the saturation limit of the surface coverage. Non detectable hybridisation signals were observed for the DNAs of wild type tobacco plant.

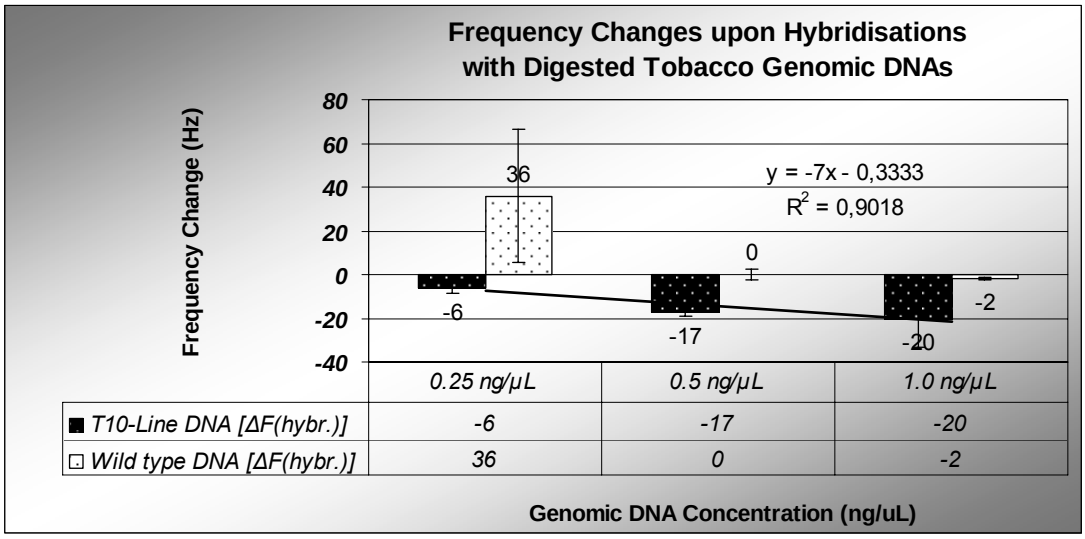

Figure 3.27. Frequency changes of QCMs upon hybridisations of P35S immobilised probe with digested (BamH1/HindIII; 16h) T10-Line *pflp* gene-inserted and wild type tobacco genomic DNAs at different concentrations.

To date, the studies based on the detection of transgenes in genetically modified crop samples via QCM - based DNA biosensors were almost only studied by an Italian group of Mascini and his co-workers (Minunni *et al.,* 2001 (a), (b); Minunni, 2003; Minunni *et al.,* 2005; Mannelli *et al.,* 2003 (a), (b), 2005). The detected samples were mostly the PCR amplified DNA samples from Certified Reference Materials (CRM) and real samples. PCR-free, direct detection of digested DNA samples were investigated by the latest studies of this group (Minunni *et al.,* 2005; Mannelli *et al.,* 2005). In those studies, hybridisation experiments were conducted with digested DNAs from real samples in order to achieve PCR - free target sequence detection and the possibility of detecting the target sequence in non-amplified DNA was demonstrated with the 10 MHz quartz crystals.

Most recently, a QCM sensor for the detection of *Cry1A(b)* gene which enhanced insecticide activity in maize was presented by Passamano and Pighini, 2006. The system was based on a calibration curve plotted by frequency decreases on a 10 MHz crystal upon hybridisations against different percentages of Certified Reference Material so that extrapolation of the frequency change gave the percentage of *Cry1A(b)* gene in that sample.

3.4.6.2. Sonicated DNAs

In the second group of experiments with the tobacco genomic DNA samples, sonication strategy was applied for the fragmentation of the DNA samples. In this study, mostly, ways to eliminate the laborious and expensive steps of developing a QCM - based DNA sensor for detecting the genetically modified organisms was surveyed since the commercialisation of the biosensors is mainly based on the practical and economical issues. Sonication of the DNA samples will not only reduce the time of the experimentation but also eliminate the use of enzymes and buffers in this manner. It is important to mention one of the major drawbacks of the system as the non-selective fragmentation of the DNA samples which may result in breaking of the surveyed transgene. So, conditions must be optimised for the sonication in order to get a minimum detection value for the hybridisation of the searched complementary sequence in the reaction medium.

A DNA amount of 5 µg was introduced into 100 µL of sterile ultrapure water (pH:8.0) resulting in a DNA concentration of 50 ng / µL for the sonication of genomic DNAs of *pflp* gene - inserted and wild type tobacco plants. The agarose gel electrophoresis profile of the fragmented DNA samples were previously illustrated in **Figures 3.10 and 3.11**. The DNAs from maximum fragmentation (90 seconds of sonication) were further diluted to 0.25, 0.5 and 1.0 ng / µL concentrations with the hybridisation buffer and checked for the hybridisation efficiency after a heat denaturation step.

Frequency changes upon hybridisations of different concentrations of sonicated genomic DNA samples of *pflp* gene - inserted and wild type tobacco plants on the P35S probe immobilised QCMs are shown in **Figure 3.28**. A similar linear relationship was observed in the frequency changes with changing DNA concentrations. An increment of frequency change was observed as the DNA concentration was increased. That result was correlated with the findings of the measurements with the digested DNAs. Moreover, the same frequency decreases were observed at the same concentrations of both digested and sonicated samples but with different standard deviations, having the sonicated samples the highest. This result might be reasonable since fragmentation of the DNAs was non - selective during sonication process which could resulted in non-specific adsorption of the DNA strands to the immobilised probes on the QCM surface. Also, at the lowest tested DNA concentration (0.25 ng / µl), a frequency decrease was also observed for the wild type tobacco plants. That freqeuncy change might also resulted from the non-specific adsoption of the DNA fragments. The detection limit was not as low in sonication procedure as the one in digestion procedure. It can be predicted that the copy number of P35S gene in digested DNA samples would be more than the sonicated samples due to the excessive shearing of DNA.

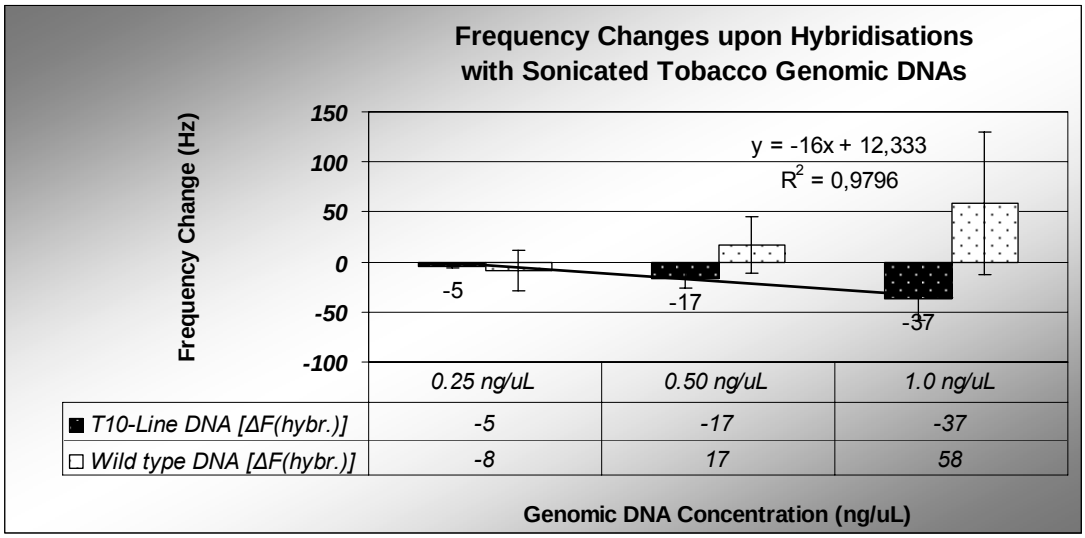

Figure 3.28. Frequency changes of QCMs upon hybridisations of P35S immobilised probe with sonicated (90 seconds) T10-Line *pflp* gene-inserted and wild type tobacco genomic DNAs at different concentrations. The line indicates the trendline according to the frequency change values of the T10-Line DNA.

The effect of the duration of the sonication process on hybridisation efficiency was further investigated. Non-sonicated and 30, 60 and 90 seconds-sonicated DNA samples were introduced into the QCM flow-cell for hybridisation. DNA concentrations of the samples were 0.5 ng/µL for the T10-Line *pflp* gene-inserted tobacco plants and 1.0 ng/µL for the negative control wild type tobacco plants. The frequency changes upon hybridisations with tobacco genomic DNAs sonicated for different durations are illustrated in the graph in **Figure 3.29**. A higher freqeuncy change was observed for the lowest duration of sonication time was detected for the transgenic DNA samples which indicated that prolonged time for sonication actually decreased the number of copies of P35S transgenes to be detected. So, 30 seconds of sonication could be suggested to be appropriate as a sonication time. Frequency changes observed from the wild type sonicated DNAs were suggested to be from non-specific adsoption to the immobilised probe.

Nonspecific adsorption of DNA leads to alterations of the measurements during both in immobilisation procedure of the probe and in the analysis of hybridisation. Especially label-free methods like QCM-based sensors are sensitive to this. The deposited mass on the immobilised sensor surface either through hybridisation with

complementary DNA sequences or interaction with non specific DNA is detected by the quartz crystal microbalance. Frequency changes with high standard deviations from the negative samples (control samples; wild type tobacco DNA) indicated that both the sonication and digestion system needed to be further optimised for the specific detection of P35S probe and target sequence hybridisation.

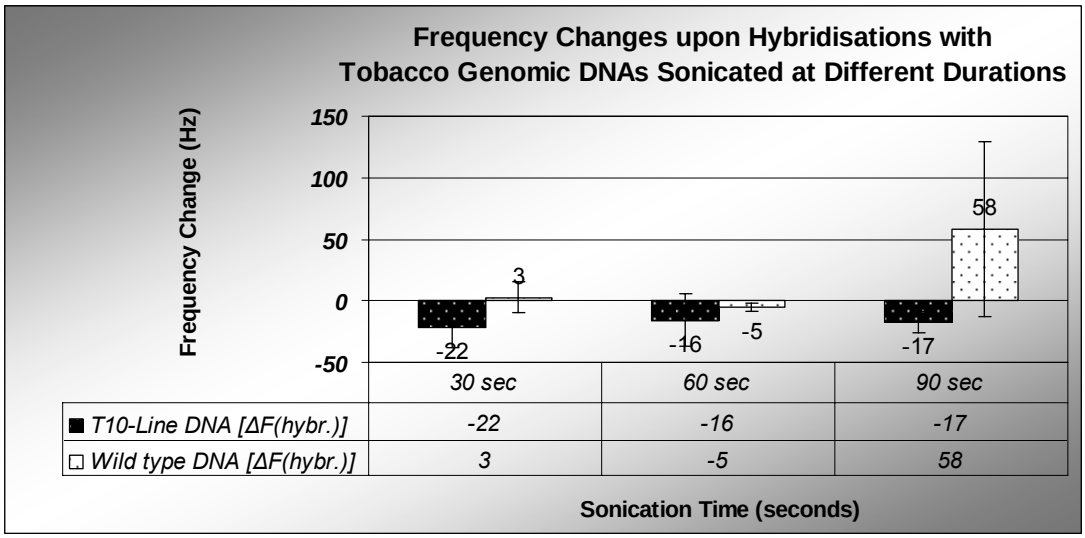

Figure 3.29. Frequency changes upon hybridisations with the T10-Line *pflp* gene-inserted and wild type tobacco plants sonicated at 30, 60 and 90 seconds. 0.5 ng/µL for the T10-Line *pflp* gene-inserted tobacco plants and 1.0 ng/µL for the wild type tobacco plants were used. Standard deviations are indicated as error bars.

CHAPTER IV

CONCLUSION

A piezoelectric QCM-based DNA biosensor for the detection of genetically modified organisms (GMOs), based on the hybridisation with the specific sequence of CaMV 35S promoter region in transgenes as a probe was developed.

The immobilisation efficiencies of chemisorption of thiolated probe on gold through thiol-gold interaction and covalent attachment of amined probe through gluteraldehyde activation of the plasma polymerised ethylenediamine layer were investigated. The thiol-derivatised probe and blocking thiol procedure was found to be efficient.

The immobilisation procedure for thiolated probe was further optimised and the values were set as follows;

- 2µM thiol-probe (25 bases) concentration,
- 2 hours exposure time, in a batch-mode, at 4°C,
- 1 hour mercaptohexanol (1mM) treatment,
- Regeneration capacity of the system was found to be 5 cycles.

The QCMs immobilised with thiolated P35S probes were firstly used for the hybridisation detection of PCR-amplified DNA samples. The lowest DNA concentration studied; 400 ng/µL, caused a frequency decrease of (- 172 Hz ± 163) upon hybridisation. Hybridisations with PCR-amplified T-Nos DNAs verified that the QCM-system was specific for P35S gene detection.

Finally, the QCM-system was studied with the digested and sonicated samples of the genomic DNAs of wild type and transgenic tobacco plants. A linear relationship was observed between the frequency changes and the hybridised DNA concentrations between 0.25-1.0 ng/µL, for both digested and sonicated transgenic samples.

The sonication process was as successful as the digestion with RE for the fragmentation of DNA samples, both giving almost the same freqeuncy shifts.

The future developments will aid the achievement of higher sensitivity and reproducibility of the biosensor.

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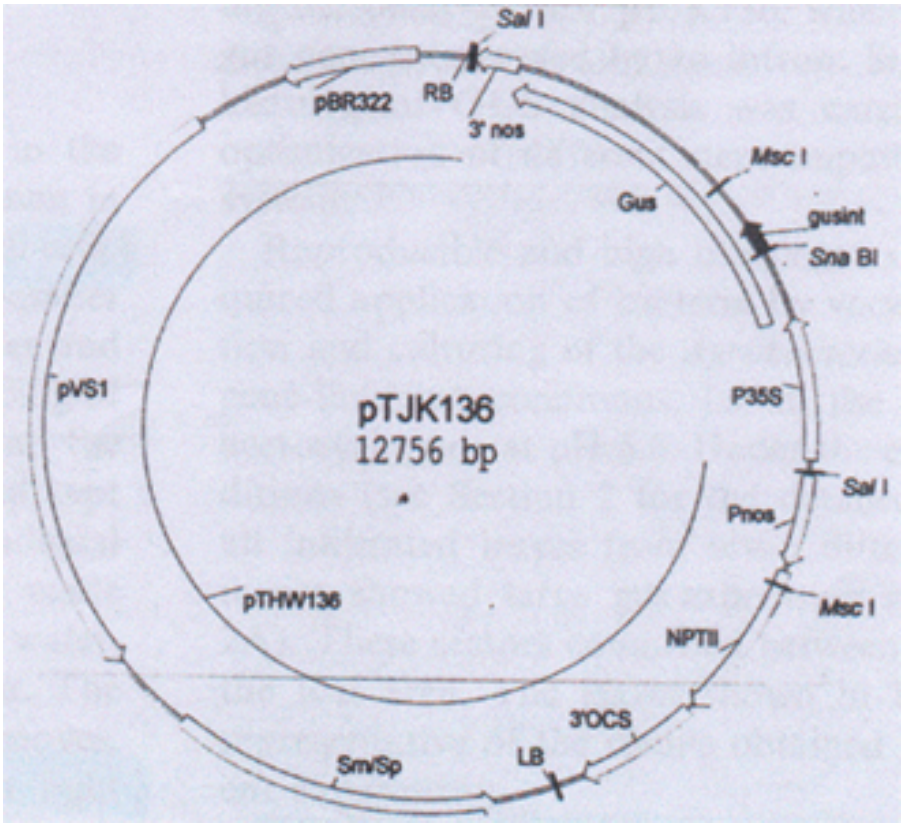
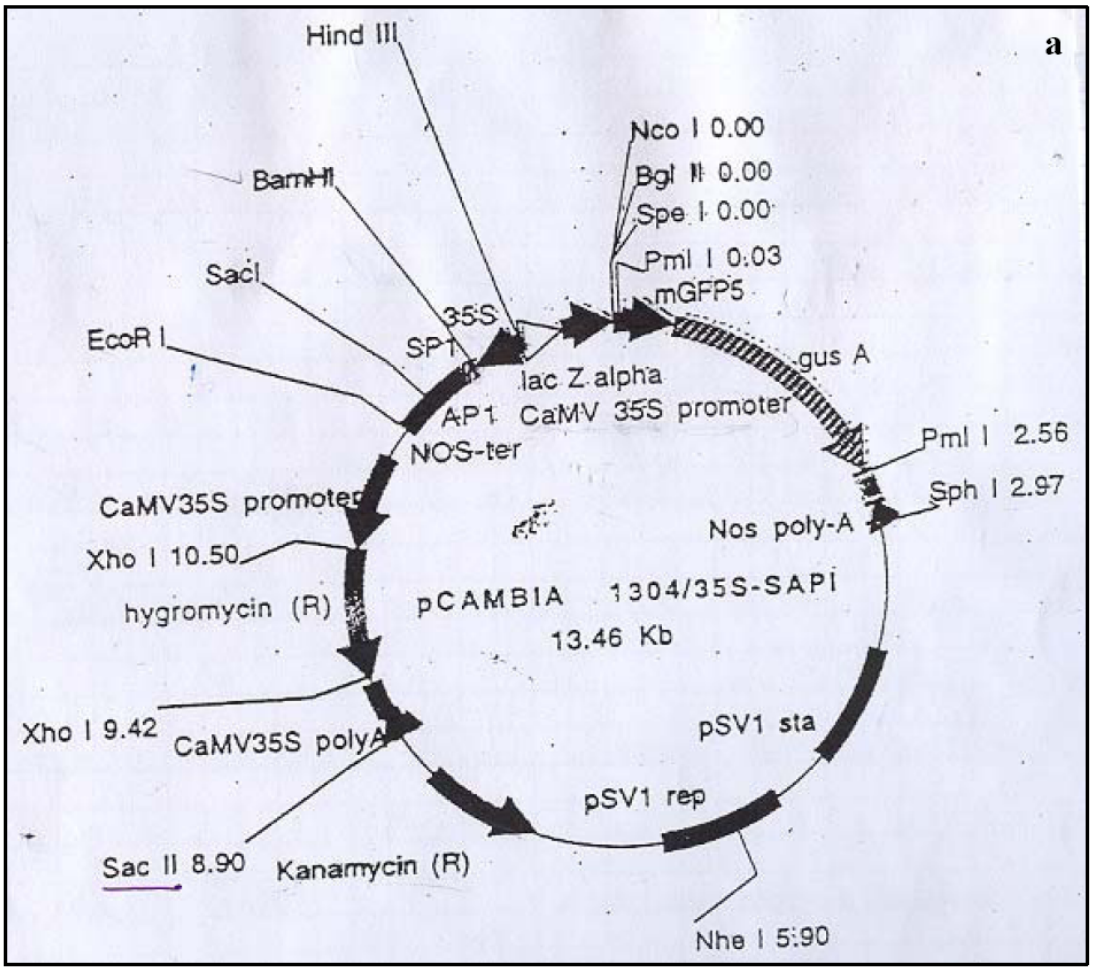
APPENDIX A

pTJK136 PLASMID DNA MAP

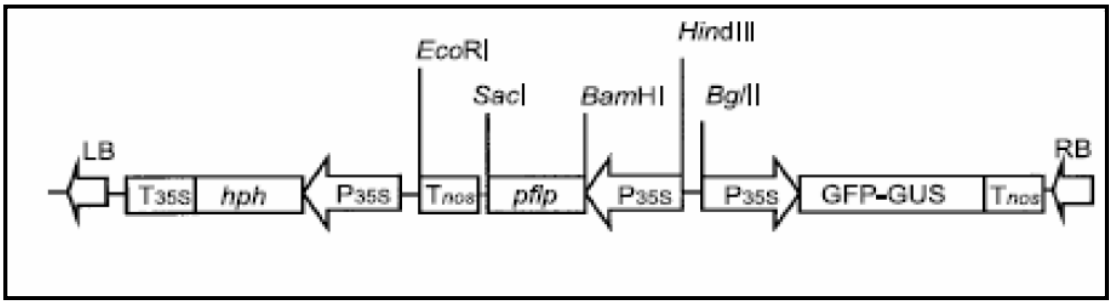


APPENDIX B

pflp PLASMID DNA MAP



T-DNA REGION of *pflp* PLASMID DNA



APPENDIX C

COMPLETE GENE SEQUENCE OF CAMV 35S PROMOTER

[Synthetic construct duplicated CaMV 35/TMV omega-prime leader sequence fusion promoter, complete sequence (*AY183361*)]

```
Primer 3 output
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER        419   20   57.64   45.00  5.00  3.00 GCTCCTACAAATGCCATCAT
RIGHT PRIMER       614   20   60.35   55.00  2.00  1.00 GGATAGTGGGATTGTGCGTC
HYB OLIGO          453   25   74.20   60.00  7.00  4.00
GGCCATCGTTGAAGATGCCTCTGCC
SEQUENCE SIZE: 735
INCLUDED REGION SIZE: 735

PRODUCT SIZE: 196, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

1 ATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCT


61 ATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCAT


121 TGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGA


181 CCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAA


241 GTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCG


301 CAAGACCCGAATTAATTCATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTC


361 GGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGC
                                                              >>

421 TCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGAC
    >>>>>>>>>>>>>>>>>              ^^^^^^^^^^^^^^^^^^^^^^^^^^

481 AGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCA


541 ACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCA
                                                        <<<<<<

601 CAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAG
    <<<<<<<<<<<<<<

661 AGGACACGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTA


721 CTATTTACAATTACA


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer
^^^^^^ hybridisation oligo
```

APPENDIX D

PARTIAL GENE SEQUENCE OF NOS TERMINATOR

[Synthetic construct transposable element dAc-I-RS DNA (*AB055064*)]

```
Primer 3 Output
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER       3925   20   62.89   55.00  4.00  0.00 GAATCCTGTTGCCGGTCTTG
RIGHT PRIMER      4104   20   58.75   45.00  6.00  6.00 TTATCCTAGTTTGCGCGCTA
HYB OLIGO         4036   25   58.94   36.00  6.00  6.00
GATTAGAGTCCCGCAATTATACATT
SEQUENCE SIZE: 7635
INCLUDED REGION SIZE: 7635

PRODUCT SIZE: 180, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 1.00


     ……….
 3841 TCAATAAGGACGAGATGGTGGAGTAAAGAAGGAGTGCGTCGAAGCAGATCGTTCAAACAT


 3901 TTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATA
                               >>>>>>>>>>>>>>>>>>>>


 3961 ATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTAT


 4021 GAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAA
                    ^^^^^^^^^^^^^^^^^^^^^^^^


 4081 AATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCG
          <<<<<<<<<<<<<<<<<<<


 4141 ATCAAACTTCGGTACTGTGTAATGACGATGAGCAATCGAGAGGCTGACTAACAAAAGGTA


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer
^^^^^^ hybridisation oligo
```

APPENDIX E

MEDIA AND SOLUTIONS USED IN COMPETENT *E.coli* CELL PREPARATION

Luria Broth (LB) Medium

2.5 g in 100 ml

pH: 7.4

SOB Medium

20g/L Tryptone

5g/L Yeast extract

0.5g/L NaCl were dissolved in 900 ml of distilled water. 10 ml of 0.25M KCl was added, pH was adjusted to 7 with 5N NaOH, the volume was completed to 1L and autoclaved. Then, 5 ml of 2M $MgCl_2$ was added under sterile conditions.

TB Solution

100 ml/L Pipes (1M)

2.21 g/L $CaCl_2.2H_2O$ (15mM)

18.64 g/L KCl (250mM) were dissolved in 800 ml of distilled water and the pH was adjusted to 6.7 with KOH. Then, 10.9 g $MnCl_2.4H_2O$ (55mM) was dissolved and the volume was completed to 1L.

APPENDIX F

SOLUTIONS AND BUFFERS USED IN MINIPREP PLASMID DNA ISOLATION

SOL I

50 mM Glucose

25 mM Tris.HCl (pH:8.0)

10mM EDTA (pH:8.0)

Autoclave and store at +4°C.

SOL II

0.2N NaOH

1% SDS

Freshly prepared.

SOL III (for 100 mL)

5M Potassium acetate	60 mL
Glacial acetic acid	11.5 mL
Distilled water	28.5 mL

TE (Tris.EDTA) (pH:8.0) (for 100 mL)

10 mM Tris.HCl [1 mL of 1M stock, pH:8.0]

1 mM EDTA [200 µL of 0.5 M stock, pH:8.0]

Complete to 100 mL with dH_2O.

Filter and sterilise by autoclaving.

APPENDIX G

BUFFERS AND SOLUTIONS USED IN QCM-BASED MEASUREMENTS

Immobilization solution II

1M KH_2PO_4, pH 3.8

Hybridisation buffer

150 mM NaCl, 20 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4

Tris-HCl (1.0 M; pH:8.0 ; 500 mL)

Tris 60.57 g

dH_2O 350 mL

Adjust pH to 8.0 with concentrated HCl (approximately 21 mL but start with less volumes).

Cool to room temperature and make final adjustments to pH.

Bring the final volume to 500 mL with dH_2O.

Filter and strelise by autoclaving.

EDTA (0.5 M; pH:8.0; 500 mL)

EDTA 93.05 g

dH_2O 350 mL

Place on a magnetic stirrer and stir vigorously.

Adjust the pH to 8.0 by adding approximately 10 g NaOH pellets.

The sodium salt of EDTA will not go into solution until the pH is adjusted to 8.0.

Bring the final volume to 500 mL with dH_2O.

Filter and sterilise by autoclaving.

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WORK EXPERIENCE

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1999 July-August	Gödöllö, Hungary	Researcher
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1995 July	Summer School of Bilimce A.Ş, Ankara	Guide Teacher

FOREIGN LANGUAGES

Advanced English, Beginner German and French

PUBLICATIONS

1. Karamollaoğlu İ., Öktem H.A., "Optimization studies for Agro-mediated transformation of Potato (*Solanum tuberosum* L.)" Abstract Book, XI. KUKEM Biotechnology Congress, Isparta, Turkey, September, 1999.
2. Karamollaoğlu İ., Yücel M, Öktem H.A. "Comparison of transgenic and wild type potatoes (*Solanum tuberosum* L. cv. Ausonia) produced by two-phase

microtuberization system" Abstract Book, Second Balkan Botanical Congress, 14-18 May 2000, İstanbul, TURKEY

3. Karamollaoğlu İ., Yücel M, Öktem H.A. "Gene transfer studies in potato (*Solanum tuberosum* L. cv Ausonia) by using *Agrobacterium tumefaciens*" Abstract Book, Second Balkan Botanical Congress, 14-18 May 2000, İstanbul, TURKEY
4. Karamollaoğlu İ. (2000) "*Agrobacterium*-mediated genetic transformation and microtuberization of potato (*Solanum tuberosum* L.)" Master of Science Thesis, Middle East Technical University, Ankara, TURKEY
5. Karamollaoğlu İ., Öktem H.A., Yücel M. "*Agrobacterium*-mediated genetic transformation of potato and production of microtubers" Poster Presentations Abstract Book, XII. Biotechnology Congress, 17-21 Oct 2001, Ayvalık, Balıkesir, TURKEY

HOBBIES

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