Ludwig-Maximilians-
Universität München

Dissertation

Analysis of paternal plastid inheritance in *Arabidopsis thaliana* and its impact on biosafety of gene-modified plants

Ashraf Elsayed

Egypt

Munich

March 2011

Ludwig-Maximilians-
Universität München

Analysis of paternal plastid inheritance in *Arabidopsis thaliana* and its impact on biosafety of gene-modified plants

Dissertation

zur Erlangung des Doktorgrades der Fakultät für Biologie der

Ludwig-Maximilians-Universität München

vorgelegt von

Ashraf Elsayed

aus Ägypten

München

März 2011

Erstgutachter: Prof. Dr. Dario Leister

Zweitgutachter: Prof. Dr. Peter Geigenberger

Tag der mündlichen Prüfung: 28.03.2011

Al-Alaq

In the name of Allah, the Beneficent, the Merciful

Read: In the name of thy Lord who createth, (1) Createth man from a clot. (2) Read: And thy Lord is the Most Bounteous, (3) Who teacheth by the pen, (4) Teacheth man that which he knew not. (5)

Summary

Genetically modified plants for the use of transgene containment are a central concern. Nuclear gene flow is one of the most discussed topics in our days; therefore, plastid genetic engineering is a promising tool to reduce the risk of transgene flow, because in most angiosperm species plastids are inherited maternally. In addition, plastid transformation has the advantage that the site of gene insertion can be controlled, high rates of transgene expression and protein accumulation can be achieved and epigenetic effects are absent. In *Arabidopsis* pollen, plastids are inherited also maternally and not created de novo, but arise from pre-existing plastids by fission.

The aim of this study was to assess the frequency of plastid transfer from atrazin-resistant *ElyF3BC4 Arabidopsis thaliana* plants bearing a point mutation in the plastid *psbA* gene to male sterile N75 plants by spontaneous crossing under field conditions. Also the plastid transfer from atrazin-resistant, EMS-mutagenized *M2ElyF3BC4* plants to wild-type *A. thaliana* plants by manual crossings under green house conditions was estimated. It was found that plastid-encoded atrazin resistance could not be transmitted via pollen, neither by manual pollination among 65,000 hybrid seeds nor by spontaneous pollination among 2,444,465 hybrid seeds in *A. thaliana*. Although various random nuclear mutations were screened for their potential to allow the transfer of paternal plastids into the egg-cells of the recipient plant, a corresponding mutant line could not be isolated. Explanation for this could be duplication or redundancy of nuclear genes mediating maternal inheritance and suppressing paternal leakage in *Arabidopsis* in such a way that the defect in one gene is compensated for by the function of its homologue. Therefore, a double mutant of two genes, *atg4a* and *atg4b*, which are involved in autophagy, were studied to test this hypothesis. However, the frequency of paternal plastid transfer was not increased. Taken together, in this study paternal leakage of *Arabidopsis* plastids could not be induced by mutations.

To be able to follow plastid fate in developing pollen tubes, the colorless plastids in *Arabidopsis* pollen were visualized by the expression of a GFP fusion protein under the control of a pollen specific promoter. However, the affiliation of the GFP labeled plastids to either the vegetative or the generative cells was not clear. Placing particular emphasis on plastid behavior during specification of sperm cells in pollen of *Arabidopsis* might shed some light on this very strict process of maternal inheritance in the future work.

Zusammenfassung

Die biologische Sicherheit von transgenen Organismen ist ein zentrales Anliegen beim Arbeiten mit genetisch modifizierten Pflanzen. Nuklearer Genfluss ist eines der am meisten diskutierten Themen der heutigen Zeit; daher ist die genetische Manipulation des Plastidengenoms ein vielversprechendes Werkzeug, um die Gefahr der Ausbreitung transgener Pflanzen zu reduzieren, da bei den meisten Angiospermen die Vererbung der Plastiden auf maternalem Wege erfolgt. Darüber hinaus hat die Transformation von Plastiden den Vorteil, dass die Geninsertionstelle kontrolliert und ein hoher Grad an transgener Expression und Proteinakkumulation erreicht werden kann und keine epigenetischen Effekte vorhanden sind. Auch im Pollen von *Arabidopsis* werden Plastiden maternal vererbt. Sie werden nicht *de novo* synthetisiert, sondern entstehen durch die Teilung bereits existierender Plastiden.

Das Ziel dieser Arbeit war es, die Häufigkeit der Plastidenvererbung von atrazinresistenten Pflanzen der *Arabidopsis thaliana* Linie ElyF3BC4, die eine Punktmutation im plastidären Gen *psbA* trägt, zur männlich sterilen Pflanzenlinie N75 bei spontanen Kreuzungen unter Feldbedinungen zu bestimmen. Des Weiteren wurde der Plastidentransfer von atrazinresistenten, EMS mutagenisierten *A.Thaliana* Pflanze der Linie M2ElyF3BC4 zu Wildtyppflanzen durch manuelles Kreuzen im Gewächshaus untersucht. Weder in den durch manuelles Bestäuben entstandenen 65.000 Samen noch in den 2.4444.465 Samen aus den Feldversuchen konnte eine Übertragung der plastidencodierten Atrazinresistenz über den Pollen festgestellt werden. Obwohl eine große Anzahl von Pflanzen mit zufälligen Mutationen im Kern auf ihr Potenzial, paternale Plastiden in die Eizelle der bestäubten Pflanze übertragen zu können, überprüft wurden, konnte keine Linie mit entsprechender Mutation isoliert werden. Gründe hierfür könnten Duplikationen oder Redundanzen kernkodierter Gene sein, die für die maternale Vererbung und die Unterdrückung der paternalen Vererbung in *Arabidopsis* verantwortlich sind. Ein möglicher Defekt könnte durch das entsprechende Homolog kompensiert werden. Um diese Hypothese zu testen, wurde eine Doppelmutante der Gene *ATG4A* und *ATG4B* untersucht, die eine entscheidende Rolle in der Autophagozytose spielen. Auch in dieses Doppelmutante war die Häufigkeit des paternalen Plastidentransfers nicht erhöht. Zusammenfassend lässt sich sagen, dass in dieser Arbeit paternale Vererbung von Plastiden in *Arabidopsis* durch Mutagenese nicht induziert oder verstärkt werden konnte.

Um das Schicksal von Plastiden während der Pollenschlauchentwicklung verfolgen zu können, wurden die farblosen Plastiden im Pollen von *Arabidopsis* durch die Expression eines GFP-Fusionsproteins unter der Kontrolle eines pollenspezifischen Promotors sichbar gemacht. Unklar blieb jedoch die Zugehörigkeit der GFP-markierten Plastiden entweder zu den vegetativen oder generativen Zellen. Legt man zukünftig einen besonderen Schwerpunkt auf die Reifung und Differenzierung der Spermazellen im Pollen von *Arabidopsis*, so könnten wichtige Einblicke in diesen sehr strikten Prozess der maternalen Vererbung gewonnen werden.

Index

Abbreviations

A. thaliana	*Arabidopsis thaliana*
AGI	*Arabidopsis* Genome Initiative
ATG	Autophagy genes
bp	Base pair
C. reinhardtii	*Chlamydomonas reinhardtii*
DNA	Deoxyribonucleic acid
dsRED	Red fluorescence protein from a Discosoma coral
DAPI	4'-6-diamidino-2-phenylindole
GMO	Genetically Modified Organism
GC	Generative Cell
PCR	Polymerase chain reaction
PMI	Polen Mitosis I
PMII	Polen Mitosis II
ptDNA	Plastid DNA
PEG	Polyethylenglycol
PFD	Photon flux density
PS II	Photosystem II
PBPI	Potential biparental plastid inheritance
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RFLP	Restriction fragment length polymorphisms
TAIR	The Arabidopsis Information Resource
T-DNA	Transfer-DNA
VC	Vegetative Cell

WT	Wild type

Units

°C	Degree Celcius
cm	Centimetre
g	Gram
h	Hour
l	Litre
µ	Micro
M	Molar
mA	Milliampere
min	Minutes
ml	Millilitre
mM	Millimolar
mol	Molar
nm	Nanometre
rpm	Rounds per minute
s	Second
V	Volt
v	Volume
w	Weight

1. Introduction

1.1 *Arabidopsis* as a Model Plant

Arabidopsis thaliana is the first higher plant whose complete genome has been sequenced (AGI, 2000; Clauss and Koch, 2006). The genome size of *Arabidopsis thaliana* is 120 megabases and the genome is organized into five chromosomes and contains about 24,000 genes. *Arabidopsis thaliana* is a small simple angiosperm plant in the mustard family (Cruciferae or Brassicaceae) that has become the model system of choice for research in plant biology. *Arabidopsis thaliana* distribute throughout Europe, Asia, and North America. The Columbia and Landsberg ecotypes (accessions) are the standards for genetic and molecular studies. The entire life cycle, including seed germination, formation of a rosette plant, bolting of the main stem, flowering, and maturation of the first seeds, is completed in 6 weeks. Flowers are composed of an outer whorl of four green sepals and inner whorls containing four white petals, six stamens bearing pollen, and a central gynoecium that forms the silique (Meinke *et al*., 1998).

1.2 Pollen Plastids

Arabidopsis thaliana pollen grains contain approximately 43 ± 15 plastids which are not visible under normal light microscope (unpigmented) because of the absence of chlorophyll. Plastids in *Arabidopsis* pollen are present in a variety of shapes while their size does not show large variations. The area of a single plastid was 1.90 ± 0.54 μm^2 (Tang *et al.* 2009). Proplastids are undifferentiated plastids in stem cells and are capable of giving rise to different plastid types and perform various cellular activities. Chloroplasts for photosynthesis are one plastid type in which thylakoid membranes are

organized. Other plastid types include, for example, amyloplasts for starch storage in roots, and chromoplasts for carotenoid in fruits (Sakamoto *et al*., 2008). Plastids are not created de novo, but arise from pre-existing plastids by fission (Aldridge *et al*., 2005). Very little is known about the division of plastids in reproductive organs including pollen grains.

Plastid genome (plastome) is present at high copy numbers (up to thousands) per single cell in land plants (Bendich, 1987).These multiple copies are packed together in large nucleoprotein bodies called nucleoids (Kuroiwa,1991). In spite of the small genome size of plastome which is typically in the range of 120 to 160 kb, in tobacco leaves 9% of the total DNA is chloroplast DNA (Tewari and Wildman, 1966). Very little is known about how plastid DNA (ptDNA) is distributed into daughter plastids upon plastid division. Plastid DNAs in higher plants do not normally undergo sexual recombination, even when they are inherited biparentally. This means that chloroplast fusion and genetic recombination does not occur in higher plants (Baldev *et al*., 1998).

Transgene containment is a central concern in genetically modified (GM) crops, especially for those which outcross to wild relatives. However, plant cells contain genomes in the nucleus, mitochondria, and chloroplasts. Whereas nuclear genes are biparentally inherited, organelle genes are in general maternally inherited (Hagemann, 2004). Therefore, engineering foreign genes in the chloroplast genome may provide containment from pollen transmission.

In angiosperms, meiosis in the anthers yields four haploid unicellular microspores. Subsequently, pollen mitosis I (PMI) yields a larger vegetative cell and a smaller generative cell (GC). The GC undergoes pollen mitosis II (PM II), a symmetric

division that yields two sperm cells. When the male gametophyte (pollen grain) meets the papillae of a receptive stigma, a complex series of cell-cell signaling events will drive pollen tube growth toward the embryo sac (female gametophyte). Upon arrival, the pollen tube tip bursts, discharging the two sperm cells. To achieve double fertilization, each sperm cell fuses with an egg or a central cell to yield the zygote and primary endosperm cell, respectively (Boavida *et al*., 2005b).

In *Arabidopsis thaliana*, PM II occurs before pollen separation (anthesis), so a tricellular mature pollen grain consists of one vegetative and two sperm cells which are later released from the anthers (Boavida *et al.*, 2005a). The vegetative cell, which makes up the bulk of a mature pollen grain, contains plastids that accumulate starch (Van Aelst *et al.*, 1993). At the initial stage of pollen formation from microspores, plastids are poorly differentiated, with an indistinguishable inner/outer membrane, in contrast to the double membrane structure of proplastids in the meristem (Robertson *et al.,* 1995; Kuang and Musgrave, 1996). Plastid differentiation and division occur alongside pollen maturation. In mature pollen, the final plastid structure contains a double membrane structure with several starch grains and simple thylakoid structures (Kuang and Musgrave, 1996). Pollen grains exist in a homogenous developmental stage in anthers, whereas the shoot meristem contains cell layers where the cells contain plastids with various morphologies and nucleoid structures (Mascarenhas, 1989 ; Fujie *et al*., 1994).

1.3 Origin of Plastid Inheritance

Only 10 years after the establishment of the Mendelian laws of inheritance and before the discovery of hereditary factors of the plastids (plastid DNA), termed plastome, Baur and Correns in 1909 simultaneously discovered and described the occurrence of

non-Mendelian inheritance of leaf variegation in *Pelargonium zonale* and *Mirabilis jalapa* plants. Although the credit for the initial observations should be shared between Baur and Correns, their subsequent theoretical interpretations distinguish their contributions to the field.

Reciprocal crosses between green and variegated plants of *Pelargonium zonale* produced F1 progeny showing various proportions of individuals with green, white and variegated leaves (Baur, 1909). The green and the white shoots of variegated plants differ in the genetic constitution of their plastids. One plastid type is green (normal, nonmutated, capable of becoming green during ontogenetic development); the other type is white (mutated, incapable of becoming green). The leaf colors of both parents were transmitted to the progeny with a bias toward the phenotype of the maternal parent. Although the ratio of transmission was essentially unequal, this phenomenon is termed biparental inheritance.

The second example was represented by reciprocal crosses between green and variegated plants of *Mirabilis jalapa* (Correns, 1909). In this case, however, leaf color of the progeny always followed that of the female parent, indicating that the trait was inherited only from the maternal parent (maternal inheritance). Correns assumed the existence of two types of cytoplasms, a healthy cytoplasm and an ill one. When (indifferent) plastids are introduced into a healthy cytoplasm, they develop into normally green chloroplasts; however, when they are introduced into an ill cytoplasm, then they remain (or become) white or yellow. Thus, the cell nuclei of the whole plant would be uniform and healthy. While the labile state of the cytoplasm switches the plant either to healthy state or to ill state.

Therefore, Baur alone deserves credit for the theory of plastid inheritance. Also Otto Renner supported Baur's theory and reported in 1930s many observations, which established plastid inheritance as a widely accepted genetic theory (Hagemann, 2000).

1.4 Modes of Plastid Inheritance

Non-Mendelian inheritance in animals usually is maternal, in contrast to the inheritance found in plants, which exhibits diversity. Plastids and their DNA can be inherited maternally, paternally or biparentally (Mogensen, 1996; Birky, 1995; Hagemann, 2004). In most species plastids are transmitted to the seed progeny by the maternal parent only. In the remaining species biparental inheritance appears to be the rule, although rare cases of paternal organelle inheritance are also known (Azhagiri and Maliga, 2007). In angiosperms, about 20% of species exhibit a strong tendancy for plastid transmission from both parent lineages (Biparental) and the remaining 80% display maternal inheritance (Zhang and Sodmergen 2010). Only a single angiosperm species, the kiwi plant (*Actinidia deliciosa*), has been found to inherit its plastids paternally (Testolin and Cipriani, 1997).

On the contrary, in gymnosperms paternal inheritance (or biparental inheritance with a strong predominance of paternal transmission) seems to be widespread (Szmidt *et al*., 1987; Neale *et al*., 1989; Mogensen, 1996), whereas *Ephedra*, *Ginkgo*, and the cycades most probably exhibit maternal inheritance of plastids (Mogensen, 1996). The sporophytic plant *Pellia*, a representative of the earliest land plants (Pacak and Szweykowska-Kulinska, 2003), a bryophyte (Natcheva and Cronberg, 2007) and two pteridophytes (Gastony and Yatskievych, 1992; Guillon and Raquin, 2000), all exhibit uniparental (maternal) plastid inheritance. In *Chlamydomonas reinhardii*, uniparental maternal inheritance is observed for the plastid genome (Kuroiwa *et al*., 1982; Nishimura *et al*., 1999).

At least in higher plants, plastid genomes do not normally undergo sexual recombination, even when they are inherited biparentally. This means that, except in very rare cases which may be considered accidents, chloroplast fusion and genetic recombination do not occur except in green alga *Chlamydomonas reinhardtii* (Medgyesy *et al*., 1985; Baldev *et al*., 1998).

1.4.1 Biparental Inheritance

Biparental inheritance does not exhibit an equal proportion of received plastids from the two parents. For instance, in Medicago, the paternal contribution is much greater and in Pelargonium, sperm and egg seem to make about equal plastid contributions to the zygote, whereas paternal plastids are even predominantly inherited in alfalfa (Hagemann, 2004). Biparental plastid inheritance correlates with (i) the distribution of microspore plastids between vegetative cell and generative cell during the first pollen mitosis, (ii) the regular presence of viable plastids in sperm cells and (iii) their entry into the zygote.

1.4.2 Maternal Inheritance

Maternal inheritance does not mean that plastid transmission from the male parent never occurs. The vast majority of angiosperms and at least some gymnosperms, bryophytes, ptridophytes and algae display a maternal mode of plastid inheritance. Electron microscopic investigations of plastid fate during male gametophyte

development distinguished four different subtypes of maternal inheritance according to the mechanism of paternal plastid elimination. The Lycopersicon type, Solanum type, Triticum type and Chlamydomonas type of maternal inheritance are named after the first species discovered to realize the respective cytological mechanism (Hagemann, 2004).

In *Chlamydomonas reinhardtii*, chloroplast DNA is transmitted maternally. During syngamy the maternal chloroplast fuses with the paternal chloroplast. Following syngamy, a zygotic maturation program leads to selective destruction of chloroplast DNA from the mt– mating type (male) parent, while the mt+ (female) chloroplast genomes survive (Nishimura *et al*., 1999). Degradation of the paternal chloroplast genomes by a specific endonuclease (Nishimura *et al*., 2002) is largely completed before fusion of the two parental chloroplasts occurs, thus resulting in uniparental inheritance of the maternal plastid DNA. Chloroplast DNA in mt+ (female) Chlamydomonas gametes is methylated by a DNA methyltransferase converting cytosine to 5- methylcytosine (Nishiyama *et al*., 2002, 2004). In contrast, plastid DNA in higher plants is nowadays believed to be unmethylated at least in somatic tissues (Marano and Carrillo, 1991; Fojtová *et al*., 2001), although some early reports had suggested that cytosine methylation can occur also in higher plant plastomes (Ngernprasirtsiri *et al*., 1988a, 1988b).

In the *Triticum* type, during fertilization of the egg cell by one of the two sperm cells, the plastids are stripped off together with most of the cytoplasm and do not enter the zygote along with the sperm cell's nucleus (Hagemann, 2004). In pea and at least some monocotyledonous species, both the generative cell and the sperm cells regularly contain plastids. These species do not transmit paternal plastids into the zygote due to degeneration of the cytoplasm surrounding the sperm cell nucleus (including plastids and mitochondria) shortly before the fertilization process.

In the *Solanum* type of maternal inheritance, plastids in the generative cells are selectively destructed whereas plastids in the vegetative cell remain intact during male gametophyte development in a number of angiosperm species. Consequently, the two sperm cells are free of plastids utilizing the plastid exclusion mechanism of the *Lycopersicon* type or utilizing the plastid degradation mechanism of the *Solanum* type.

The *Lycopersicon* type of maternal plastid inheritance involves plastid exclusion during the first pollen mitosis. The extremely asymmetric division of the microspore results in a vegetative cell that contains all plastids and a generative cell that is free of plastids. Consequently, both sperm cells lack plastids. It is generally assumed that plastid inheritance in the majority of angiosperm species follows the exclusion mechanism of the *Lycopersicon* type. *Arabidopsis* belongs to the *Lycopersicon* type (Hagemann, 2004).

1.4.3 Paternal Inheritance type and Paternal Leakage

Electron microscopic investigations confirmed the absence of plastids from egg cells and the presence of them in sperm cells in gymnosperm species displaying paternal plastid inheritance. Two distinct mechanisms can contribute to paternal inheritance: plastid exclusion by unequal organelle distribution during female gametophyte development and/or plastid degradation in the egg cell (Mogensen, 1996; Hagemann, 2004).

Distinction between purely paternal inheritance and biparental inheritance has been difficult, because most studies on plastid inheritance in gymnosperms suffer from statistically reliable datasets. This is due to the lack of suitable phenotypic markers,

which limit the number of progeny seedlings that can be analyzed and makes it difficult to exclude maternal plastid transmission below a certain level (Hagemann, 2004). It seems reasonable to suspect that the rate of paternal leakage can be very different in species representing the different subtypes of maternal inheritance, but this remains to be established experimentally.

In higher plants, confirmation of uniparental–maternal inheritance of plastids in reciprocal crosses was usually obtained by the study of a small number of plants up to about 100. However, when progeny size was significantly increased to include 1000–2000000 plants, rare exceptions to maternal inheritance could be obtained. The examples include *Antirrhinum majus* (Diers, 1967), *Petunia hybrida* (Cornu and Dulieu, 1988; Derepas and Dulieu, 1992), *Nicotiana tabacum* (Avni and Edelman, 1991; Horlow *et al*., 1990; Ruf *et al*., 2007; Svab and Maliga, 2007), and *Setaria italic* (Wang *et al*., 2004). In addition, more extensive studies led to the realization that significant variability exists within a species with respect to the frequency of paternal plastid transmission. In *Chlorophytum comosum*, a species known to display biparental inheritance, paternal plastids are inherited at a rate of 2–8% (Pandey and Blaydes, 1957). In *Petunia hybrida* frequent (0.1–2%) transmission of paternal plastids could be readily detected in six out of 22 inbred lines (Cornu and Dulieu, 1988; Derepas and Dulieu, 1992).

Reliable quantitative data came only from two genetic studys in *Setaria italica* and *Arabidopsis thaliana*. In foxtail millet, *Setaria italica*, crosses between male-sterile yellow- or green-leafed herbicide susceptible lines (as maternal parent) and a line with chloroplast-inherited atrazine resistance as pollen donor were employed (Wang *et al*., 2004). Assaying more than 780,000 hybrid offspring for atrazine resistance as it would be caused by paternally transmitted plastid genomes, paternal leakage was detected at a frequency of $3x10^{-4}$. In *Arabidopsis thaliana*, crosses between the male sterile

spectinomycin susceptible line Ler-ms1-1 as maternal parent and chloroplast inherited spectinomycin resistant fertile mutant RLD-spec1 as pollen donor were performed (Azhagiri and Maliga, 2007). Only three spectinomycin-resistant calli were identified among 76 825 calli (3.9×10^{-5}) selected on spectinomycin-containing ARMI callus induction medium. This observation extends previous reports to a cruciferous species suggesting that low-frequency paternal leakage of plastids via pollen may be universal in plants previously thought to exhibit strict maternal plastid inheritance.

1.5 Screening and Visualizaion of Plastids in Pollen

The study of organelle inheritance began with the use of phenotypic markers in genetic study and has recently been extended by molecular and cytological approaches. Because the disadvantages of one technique can often be overcome by the use of another, recent studies often combine several approaches. The markers employed in plastid inheritance studies were leaf pigment mutations, that were readily apparent as variegated sectors (Cornu and Dulieu, 1988; Diers, 1967). Other plastome mutations including resistance to antibiotics selectable in culture (Avni and Edelman, 1991; Horlow *et al*., 1990; Medgyesy *et al*., 1986; Ruf *et al*., 2007; Svab and Maliga, 2007; Azhagiri and Maliga, 2007), herbicide resistance mutations that could be tested in the field (Wang *et al*., 2004) have been utilized as markers for studying plastid inheritance.

The laborious and time-consuming genetic analyses required to find out the mechanism of plastid inheritance or to establish low level paternal leakage make it desirable to develop faster assays suitable to assess a species' potential to occasionally transmit paternal plastids via pollen. Rapid screening method employs electron

microscopy and the use of DAPI 4'-6-diamidino-2-phenylindole. DAPI stains intensely plastid nucleoids which then can be readily detected by fluorescence microscopy. Absence of stainable plastid DNA from generative and sperm cells was taken as evidence for strictly maternal inheritance, whereas species with detectable ptDNA in generative and/or sperm cells were classified as potential biparental plastid inheritance (Nagata *et al.* 1999).

The presence of plastid DNA in male gametic cells is a prerequisite for paternal transmission. This phenomenon is called potential biparental plastid inheritance (PBPI). Experimentally, all species exhibiting biparental plastid inheritance in genetic analyses show PBPI (Kuroiwa, 1991). Conversely, however, in very few cases, plants with PBPI may not be identified genetically as showing biparental plastid inheritance. This might be because PBPI is sometimes weak (Zhang and Sodmergen 2010) and paternal transmission cannot be easily traced when a very small proportion of male plastid DNA is contributed. Therefore, PBPI is a cellular indicator, and is possibly a more sensitive and accurate than genetic analysis for biparental plastid inheritance. Also, GFP-labeled plastids in *Arabidopsis* pollen are an effective method to directly detect plastids (Tang *et al*., 2009).

Recently, molecular techniques have allowed restriction fragment length polymorphisms (RFLPs) to be used as specific markers for organelle DNA. This technique is based on the digestion of organelle DNA with restriction endonucleases, which reveals genotype-specific patterns when cleavage products are separated electrophoretically. Typically, total cellular DNA is extracted, and then organelle DNA is identified with isolation of specific gene for organelle using PCR (polymerase chain reaction) amplification of organelle DNA technique, followed by RFLP analysis of the amplification products permits an increased detection rate of parental organelle DNA.

Once distinct organelle DNA genotypes are recognized by mapping the restriction fragment patterns, they can be utilized to identify the parental origin of the organelle DNA in hybrid plants by comparing the patterns of each parent with those of the progeny. This approach has been used in studies of plastid DNA inheritance and evolution in Iris. Plastid transmission is very likely controlled by nuclear genes, but to date, not a single gene involved in plastid inheritance has been identified in higher plants (Azhagiri and Maliga, 2007).

1.6 Autophagy and Plastid Inheritance

Autophagy is generally defined as a lysosome-dependent mechanism of intracellular degradation that is used for the protein turnover of cytoplasm. Several forms of autophagy have been described, including macroautophagy and microautophagy (Xie and Klionsky, 2007). Both microautophagy and macroautophagy are functional in plants (Bassham *et al*., 2006). In plant microautophagy, the target material is directly engulfed by an invagination of the tonoplast.

Macroautophagy (hereafter “autophagy”) in plants is a process that starts with the formation of cup-shaped membranes in the cytoplasm. After completion, autophagosomes have at least two destinations in plants. They may fuse with the tonoplast and be directly delivered to the lumen of the vacuole as seen in *Arabidopsis*. Alternatively, autophagosomes may first transform into lysosome-like acidic and lytic structures and, fusion with the central vacuole may occur as a secondary event (Toyooka *et al*., 2006; Inoue *et al*., 2006).

Macroautophagy occurs at basal levels in growing cells, allowing them to recycle long-lived proteins and organelles (Klionsky, 2005). The cargo is degraded into its building blocks (i.e., proteins to amino acids), helping the cell to economize its resources, eliminate old/damaged organelles, and survive nutrient and other types of stress (Mitou *et al*., 2009).

In the model plant *Arabidopsis thaliana*, 25 orthologs of 12 yeast ATG genes were identified (Mitou *et al*., 2009). Some exist as a single copy (i.e., Atg3 and Atg5) and others as multiple copies (i.e., Atg1 and Atg8). Functional domains of these *Arabidopsis* proteins were well conserved during evolution, indicating preservation of basic autophagy mechanisms in plants. Indeed, complementation tests in ATG mutant yeast strains using some of the plant Atg proteins confirmed the preservation of their function (Ketelaar *et al*., 2004).

The pollen of *Arabidopsis* is of the tri-cellular type, which means the generative cell divides again to form two sperm cells before pollen germination. In microspores during the formation of intine (inner cell wall), a large vacuole appeared which was made by fusion of pre-existing vacuoles and probably absorption of solutions. In the young pollen grain after the first mitosis, a large vacuole was divided into small vacuoles. After the second mitotic division, vacuoles with the similar appearance to those in the previous stage were no longer observed. The autolysis of mature pollen grains may contribute to the self pollination in *Arabidopsis thaliana* (Yamamoto *et al*., 2003).

1.7 Aim of the Thesis

This study has three aims:

1. To estimate the frequency of paternal leakage of plastids via pollen under field conditions in *Arabidopsis thaliana*.
2. To search for mutant plants in *Arabidopsis thaliana* which show enhanced ability for plastid transmission via pollen. Therefore a genetic screening approach was established.
3. To test if autophagy is involved in maternal inheritance of plastids in *Arabidopsis thaliana*.

2 Materials and Methods

2.1 Plant Materials

Ely accession was purchased from the *Arabidopsis* stock centre NASC (HTTP:// ARABIDOPSIS.INFO/). These seeds which were collected at the railway station of Ely (UK) in 1988 are atrazin-resistant mutant due to a mutation in the plastid *psbA* gene. The mutation is a nucleotide exchange in *psbA* gene encoding the D1 protein at position 264 from AGT to GGT codon in the *Arabidopsis* ptDNA. Ely has *Ler* nuclear back-ground. The ElyF3BC4 seeds were kindly provided by Prof. Koornneef (El-Lithy *et al.*, 2005). F3BC4 plants were obtained by crossing Ely (female parent) x Ler (male parent). Resulting F1 plants were crossed again with *Ler* as the male parents to get the 2nd back cross (BC). The same was done for two further generations until 4th BC plants were obtained. These plants were selfed two times to get F3 plants of the 4th BC (F3BC4). The aim was to obtain plants with *Ler* nuclear DNA and Ely cytoplasm.

Mutagenization of ElyF3BC4 seeds via EMS (Ethanmethylsulfonate) mutagenesis to produce M1 seeds were purchased in USA at Lehle Seeds Company (HTTP://WWW.ARABIDOPSIS.COM/). The M1 seeds were germinated and selfed in the same company to produce M2ElyF3BC4 seeds. The M2ElyF3BC4 seeds have thousands random mutations showing high mutation frequency estimated by Mednik´s P value (P= 98/100).

Purchased N75 seeds from NASC are nuclear male sterile mutant seeds (ms1), which are characterized by the lack of viable pollen due to transition from G^{882} to A^{882} at exon2–intron2 junction in the *MS1* gene that leads to a mis-spliced transcript (Wilson *et al.,* 2001).

The double mutant atg4aatg4b is autophagy-deficient mutant, due to T-DNA insertion in ATG4a (at2g44140) and ATG4b (at3g59950). Seeds were kindly provided by Prof. Ohsumi (Yoshimoto *et al.,* 2004).

2.2 Seeds Sterilization

Arabidopsis seeds were purified from non-seed particles by sieving. Surface sterilization of seeds was performed by gas sterilization under hood (5% Conc.HCl in Sodiumhypochloride 37%). The sterilization period depends on the quantity of seeds, but generally, it took from three hours in case of few seeds and five hors in case of abundant seeds.

2.3 Growth Conditions in Soil

All *Arabidopsis* seeds were sown in pots with standard soil, followed by cold treatment (vernalization) for 2 d at 4 °C in dark to break dormancy and to synchronize germination. Then pots were moved to a temperature controlled greenhouse controlled, 70-90 µE m^{-2} s^{-1} illuminations with a day-night cycle of 16 h/ 8 h for 2 weeks. Then plants were replanted in 54 wells tray with standard soil. Fertilization with “Osmocote Plus” (Scotts Deutschland GmbH, Nordon Germany) was performed according to manufacturer’s instructions. Then plants grown another 4-6 weeks in the same conditions till flowering, and seeds were harvested.

2.4 Growth Conditions on ms-plates

Gas-sterilized seeds were spread on a solid Murashige and Skoog (MS) medium under aseptic conditions. MS-medium contained salts, vitamins, 1% sucrose, 0.8% agar. Vernalization of sterilized seeds was at 4 °C for 2 days in dark to break dormancy and to

synchronize germination was performed. Afterwards seeds were grown on plates in growth chamber under 80 $\mu Em^{-2}s^{-1}$ illuminations with a day-night cycle of 16 h: 8 h. Screening of plants on 1x MS medium containing 10 µM atrazin was done, and resistance to atrazin was manifested as green seedlings.

2.5 Spontaneous Out-crossing in Field

The first field experiment was in 2008 and the second field experiment was in 2009. The setup of the two field experiments was arranged in 3 different field areas: (46, 2 m²; 15, 4 m²; 24, 6 m²) at Rostock University by the frame of Prof. Broer (Figure 1). Spontaneous out-crossings among different *Arabidopsis* accessions N75, Ely, *Ler* and *Col-0* were measured.

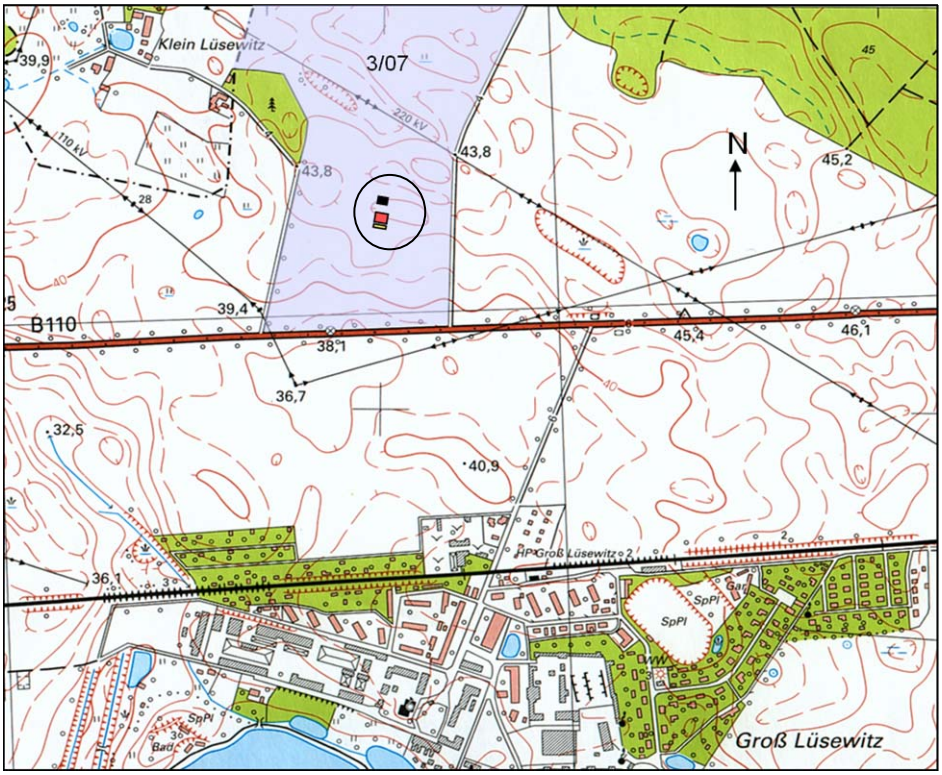

Figure 1. Map of Field Areas used in out-crossing experiments. the location of field trials experiment measuring spontaneous paternal plastid inheritance in *Arabidopsis*. The circle enclosing field areas at Rostock University.

Trial 1: 6,00 m x 7,70 m = 46,2 m², Trial 2: 2,00 m x 7,70 m = 15,4 m² Trial 3: 6,00 m x 4,10 m = 24,6 m²

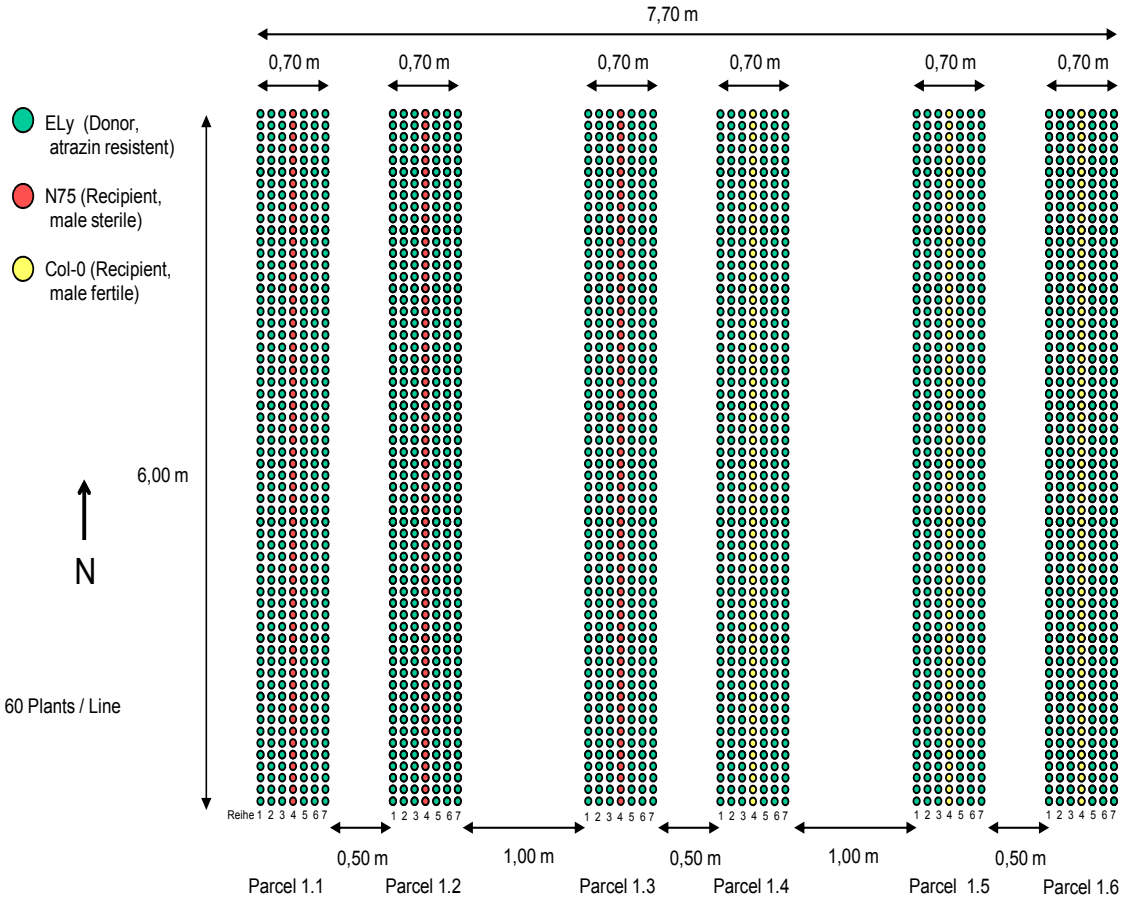

Figure 2. High Pollen Pressure in field out-crossing experiment. In 2008, ElyF3BC4 plant was the pollen donor and both N75 and Col-0 was the pollen recipient. In 2009, ElyF3BC4 plant was the pollen donor and N75 was the pollen recipient. ElyF3BC4 represented as green circles, N75 as red and Col-0 as yellow.

High pollen pressure was applied in the first trial of field experiment as illustrated in Figure 2. There were 6 parcels separated by 0.50 m and 1.00 m distances and each parcel consisted of 7 rows, each row has 60 plants. In the high-pollen-pressure experiment, there was only one row of pollen recipient plants in the middle (number 4) of

each parcel, surrounded by 6 rows (1, 2, 3, 5, 6 and 7) of pollen donor Ely plants. N75 was the pollen recipient plant in parcels 1.1, 1.2 and 1.3, whereas *Col-0* was the pollen recipient plant in parcels 1.4, 1.5 and 1.6.

***Arabidopsis* Field Trial 2.**

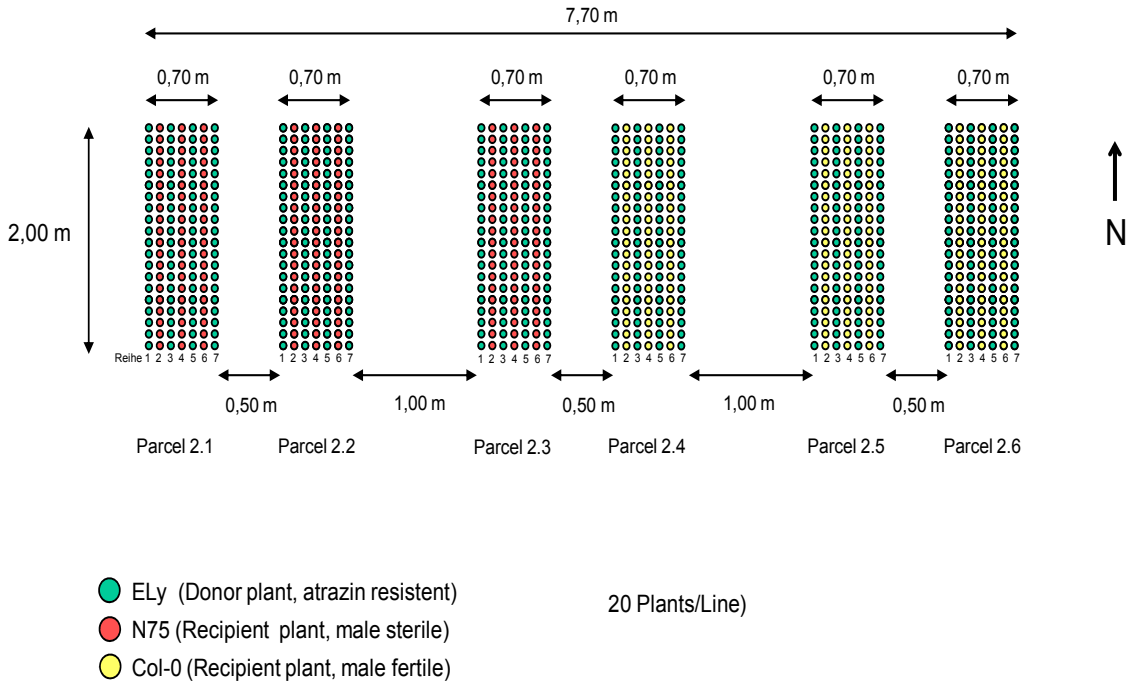

Figure 3. Low Pollen Pressure in field out-crossing experiment. In 2008, ElyF3BC4 plant was the pollen donor and both N75 and *Col-0* was the pollen recipient. In 2009, ElyF3BC4 plant was the pollen donor and N75 was the pollen recipient. ElyF3BC4 represented as green circles, N75 as red and *Col-0* as yellow.

The low pollen pressure was applied in the second trial of field experiment as illustrated in Figure 3. There were 6 parcels separated by 0.50 m and 1.00 m distances

and each parcel consists of 7 rows, each row 20 plants. The pollen donor plants Ely (in rows 1,3,5 and 7) and pollen recipient N75 and *Col-0* plants (in rows 2,4 an 6) were cultivated in an alternative way or in another words one row pollen donor plants sided by one row pollen recipient plant. N75 was the pollen recipient plant in the first three parcels 2.1, 2.2 and 2.3, while *Col-0* was the pollen recipient plant in the last three parcels 2.4, 2.5 and 2.6.

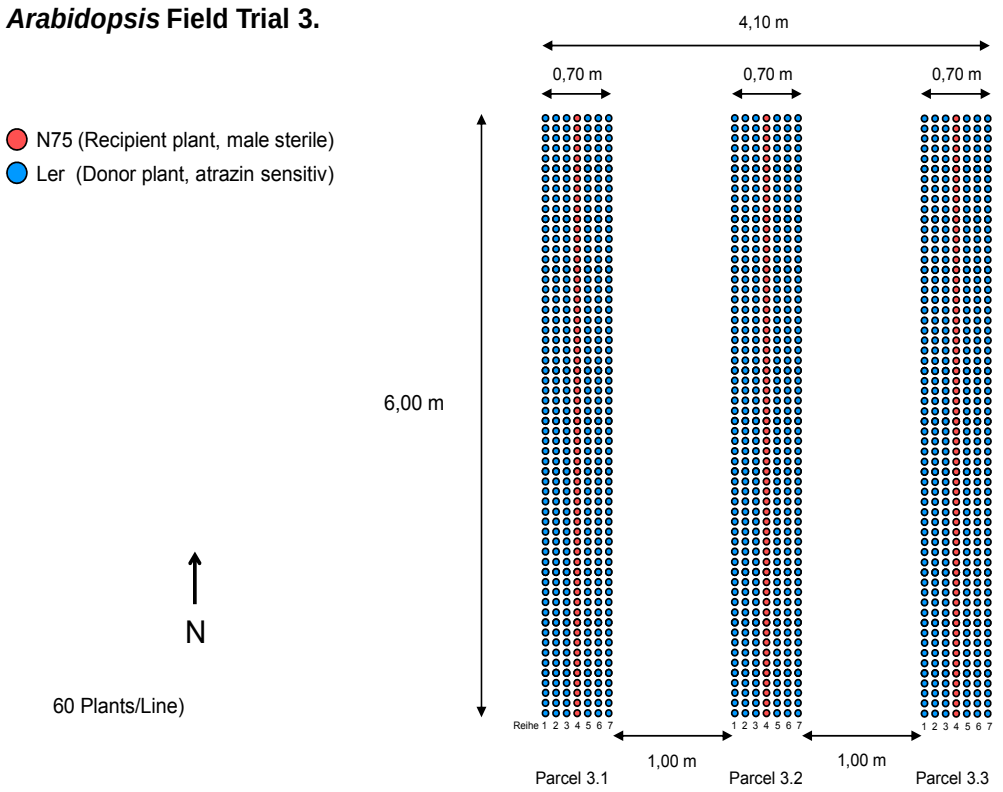

Figure 4. Control in field out-crossing experiment. In 2008 and 2009, *Ler* plant was the pollen donor N75 was the pollen recipient. N75 as red circles and *Ler* as blue.

The control field experiment in the third trial was designed as in Figure 4. It consists of 3 parcels 3.1, 3.2 and 3.3 separated by 1.00 m distances among them, each parcel consists of 7 rows and each row has 60 plants. *Ler* plants were the pollen donor in rows (1, 2, 3, 5, 6 and 7) in all parcels surrounding the single pollen acceptor N75 plants in the middle row.

2.6 Manual Crosses in Greenhouse

It is convenient to do the manual pollination in the early morning, where shedding of pollens from anthers starts in the morning in most plants. Also, high humidity is a condition for getting successful crosses because high humidity is necessary for pollen germination. Anthers in pollen recipient *Ler* plants were emasculated from closed flowers by dissection under dissecting microscope using crossing forceps with narrow tips. First hand the inflorescence gently and hold the forceps by the second hand, so that the forceps is perpendicular to the pistil. Then, sepals, petals and stamens were removed from closed flowers of egg-donor *Ler* plants by cutting and not by pulling. Forceps tips are working like scissor when getting gentle pressure on them. Stigmas in emasculated flowers were pollinated manually by pollens from M2ElyF3BC4 flowers. Manual pollination happened, when gentle touch between stamens in opened flowers (pollen donor plants) and naked stigma in emasculated flower (pollen recipient plants) achieved.

2.7 Molecular Analysis

2.7.1 DNA Extraction

Arabidopsis genomic DNA was isolated from plant leaves by homogenization in extraction buffer: 200 mM Tris-HCl (pH 8.0), 250 mM EDTA250 mM NaCl, 0.5 % SDS. The homogenate was incubated for 15 min at room temperature. After centrifuging for 5

min 16000 xg at 23 °C speed, an equal amount of cold isopropanol was added to precipitate the DNA by carefully inverting the tube several times. After 5 min, 16000 xg speed at 23 °C. The water–alcohol mixture was discarded and the pellet washed with 70% cold ethanol. The pellet was left to dry and dissolved in water containing RNase A and incubated for 30 min at 37 °C. Thereafter it was stored at 4 °C.

2.7.2 PCR –RFLP Analysis of Plastid DNA

478 bp plastid DNA sequence from the *psbA* gene flanking the mutation area at codon position 264 , was amplified from total genomic DNA isolated from fresh leaves by PCR (Polymerase Chain Reaction) using the primers D1 forward 5‘-GGA ATC TCT GGT ACT TTC AAC TTT-3‘ and D1 reverse 5‘-GTT CAT GCA TAA CTT CCA TAC CAA -*3*“. PCR was carried in a volume 20 µl PCR reaction mixture containing 2.0 µl DNA, 1.0 µl D1-Forward primer, 1.0 µl D1-Reverse primer, 2.0 µl 10x PCR-buffer, 2.0 µl dNTPs (2 mM), 1.2 µl MgCl2 (25 mM), 0.3 µl Taq-polymerase and 10.5 µl water. Amplification was carried out using a Bio-Rad Cycler Thermal Cycler (Bio-Rad,USA) with 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 m at 72 °C . The PCR product were purified using QIAquick PCR Kit (Qiagen Inc., www.qiagen.com) before DNA digest and before sequencing.

RFLP analysis was achieved by cleaving the PCR product of *psbA* gene by BstXI restriction enzymes. The recognition site for BstXI was flanking the mutated codon in the amplified sequence of *psbA* gene. 1x buffer and incubation at 37 °C for 1-2 hours were used for optimal reaction conditions. The restriction fragments were separated by electrophoresis.

2.7.3 *MS1* Sequencing

506 bp nuclear DNA sequence from *MS1* gene flanking the mutation area at base position 882 , was amplified from total genomic DNA isolated from fresh leaves by PCR using the primers MS1- Forward 5‘-GAA CCT TGC AGG TTG GGG AAA TC -3‘ and MS1- Reverse 5‘-GCA GCA GCC TCA ACT CCA TTC -*3“*. DNA sequencing of *MS*1 gene was performed using the primers that were used for DNA amplification at the sequencing service at the LMU München (HTTP://WWW.GENETIK.BIOLOGIE.UNI-MUENCHEN.DE/SEQUENCING). DNA samples were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit and purified samples were analyzed on an ABI 3730 48 capillary sequencer with 50 cm capillary length.

2.7.4 PCR Analysis of atg4aatg4b double mutant

In homozygosity check for atg4aatg4b double mutant, 10 PCR reactions amplifying ATG4s from genomic DNA of double autophagy mutant atg4a4b were carried out using a Bio-Rad Cycler Thermal Cycler (Bio-Rad,USA) with 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 2.30 m at 72 °C . Homozygosity test for ATG4a gene, was performed by 5 PCR reactions using a combination of T-DNA specific primers (PGAP-1 and PGAP-2) and ATG4a specific primers (ATG4a-Forward and ATG4a-Reverse).Primer sequences were as following : PGAP-1: 5‘- CAT TTT ATA ATA ACG CTG CGG ACA TCT AC -*3“* , PGAP-2: 5‘-TTT CTC CAT ATT GAC CAT CAT ACT CAT TG-*3“* , ATG4a-Forward : 5‘- ATG AAG GCT TTA TGT GAT AGA TTT GTT C-*3“* and ATG4a-Reverse 5‘- TCA GAG CAT TTG CCA GTC ATC TTC AC-*3“*.

Another 5 PCR reactions for ATG4b gene, were done by using a combination of T-DNA specific primers (PGAP-3 and PGAP-4) and ATG4b specific primers (ATG4b-Forward and ATG4b-Reverse). Primer sequences were as following : PGAP-3: 5‘- TAG

ATC CGA AAC TAT CAG TG -*3*“ , PGAP-4: 5‘-ATA ACG CTG CGG ACA TCT AC-*3*“ , ATG4b-Forward : 5‘- ACA GAC ATG TTG TAT TTG GTG CTT AAT GA-*3*“ and ATG4b-Reverse 5‘- GTC ACA CAA TGA AAA GAA TGG CTA GGA G-*3*“. PCR products were analysed on 1% agarose gel by electrophoresis.

2.7.5 Agarose Gel Electrophoresis

The restriction fragments were separated by electrophoresis. 1% agarose gel in 1x TBE-buffer for large DNA fragments and 2% agarose gel in 1x TBE-buffer for small DNA fragments. Agarose gels were stained with ethidium bromide and visualized on UV light and photographed.

2.7.6 Enzymatic Manipulation

All enzymatic manipulations (ligation, restriction and dephosphorylation) were done according to the included manual instructions of the supplier.

2.7.7 DNA Constructs

2.7.7.1 STP9.GFP and STP9.TP.GFP Constructs

STP9.GFP construct in pLEX7 plasmid was donated by Prof. Büttner (Schneidereit *et al*., 2003). STP9.GFP was cloned into EcoRI and HindIII sites of pGREEN II (http://www.pgreen.ac.uk/). For creating the STP9.TP.GFP construct, a 300 bp transit peptide fragment corresponding to the 5-end of At5g54800 open reading frame carrying NcoI site at both sides was amplified by PCR using primers pd2-F: *5‘*--CCC GGG CCA TGG TTTT ATC GGT GAA GC -3‘ and pd2-R *5‘*- – CCG GCC ATG GGG TAA ATG CCG ATC TTC AAT TTC -3‘. The PCR product was cut by NcoI and introduced into pLEX7 (STP9p_pEP) plasmid carrying GFP under the control of the Stp9

promotor using NcoI site. Afterwards, STP9.TP.GFP construct cloned into ECoRI and HindIII sites of pGREENII vector.

2.7.7.2 STP9.RFP.AAP3 Construct

The red fluorescent protein (RFP) from the reef coral Discosoma (dsRED) (Jach et al., 2001) was used as a reporter to label the plasma membrane in *Arabidopsis* polen. This was accomplished by a cloning strategy based on the GATEWAY Technology (Invitrogen) using the primersFor constructing STP9.RFP.AAP3 (1592bp), 666 bp pollen promoter from At1g50310 (stp9) carrying TOPO site at *5'*-end and NcoI site at 3'-end was amplified by PCR using the primers stp9/topo-s *5'*-CAC CAG AGA ACG TAC GGT GTG TTC ATA A-3' and stp9/NcoI-as *5'*-CCA TGG TAT TAT TTA TTC TTC ACT TAT TG-3' and cloned into an entry vector (pENTR ™ /D-TOPO®) by TOPO cloning reaction. Afterwards 671 bp from RFP (pGj1425) carrying NcoI site at *5'*-end and EcoRI-NcoI site at 3'-end was amplified by PCR using the primers RFP/NcoI-s *5'*-CCA TGG GGT CTT CCA AGA ATG TTA TC-3' and RFP/EcoRI/NcoI-as *5'*-CCA TGG GAA TTC AAG GAA CAG ATG GTG GCG-3' and introduced into pENTR ™ /D-TOPO® vector at NcoI site. The third insert was 255 bp from AAP3 sequence which carrying EcoRI site at both end primes was amplified by PCR using the primers AAP3/EcoRI-s *5'*-GAA TTC GGG GGA GGC GGA GGG ATG GTT CAA AAC CAC CAA ACA GTT CTG G -3' and AAP3/EcoRI-as *5'*-GAA TTC TAA GTG ACG GCA GAG AAG AGC AAC-3'. The TOPO vector containing the construct STP9.RFP.AAP3 was amplified. Then stp9.rfp.aap3 fragment was subsequently subcloned into the distination vector pP001-VS-GW by LR Clonase II enzyme.

2.7.8 Transformation of *E.coli*

GFP-construct and RFP-construct were used to transform DH5α competent *E.coli* bacterial cells by the heat shock method 90 seconds at 42 °C. Competent bacterial cells DH5α were prepared by cold 0.1M CaCl2 treatment. Transformed *E.coli* lines by GFP constructs were screened with kanamycin antibiotic, while Transformed *E.coli* lines by RFP constructs were screened with ampicillin antibiotic.

2.7.9 Plasmid Preparation

Plasmid preparations of GFP-construct and RFP-constructs were performed by QIAquick miniprep Kit according to the included manual instructions (Qiagen Inc., www.qiagen.com).

2.7.10 Transformation of Agrobacterium

STP9.GFP and STP9.TP.GFP constructs in pGREENII vector were transformed into electro-comptent Agrobacterium strain GV3101 with the help of pSOUP vector which was necessary for replication. Also, STP9.RFP.AAP3 Construct was transformed in the same electro-comptent Agrobacterium strain GV3101, but without pSOUP vector. Screening was performed as mentioned above in (chapter 2.7.8)

2.7.11 Agrobacterium-mediated transformation of *A. thaliana*

GFP-construct and RFP-constructs were used to transform Col-0 Arabidopsis plants as described by Clough and Bent (1998). Flowering plants were dipped for 15 s in the appropriate Agrobacterium suspension containing 5% sucrose and the surfactant Silwet L-77 (0.0005%). After dipping, plants were transferred to the greenhouse and seeds were collected after approximately 3 weeks. In vitro selection for resistance either to kanamycin (GFP lines) or to BASTA herbicide (RFP lines) was carried out and the

transgenic plants were grown on soil under greenhouse controlled conditions (PDF: 70-90 µEm-2s-1, 16h light: 8h dark cycles). The integration of the transgene in the genome of the resistant plants was confirmed by PCR, using the vector specific primer in combination with the gene specific one.

2.8 Pollen Germination *In vitro*

Fresh anthers from opened flowers in plants carrying GFP-fusion protein and RFP-fusion proteins besides anthers from WT *Col-0* plants were used for pollen germination *in vitro*. The dehisced anthers of three randomly picked inflorescences were carefully dipped onto the surface of agar plates to transfer the pollen grains. The Basic Medium for *in vitro* pollen germination contained 5 mM MES (pH 5.8 adjusted with TRIS), 1mM KCl, 10 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.5 mM boric acid, 1% (w/v) agar (K^+-depleted agar), 16.6% (w/v) sucrose, 3.65% (w/v) sorbitol, and 10 µg ml $^{-1}$ myo-inositol. The medium was prepared with double-distilled water and heated to 100 °C for 2 min. Each agar slide contained 0.5 ml medium forming a thin layer. Following pollen application, the slides were immediately transferred to a chamber at 25 °C with 100% relative humidity in the light 30 µ mol m^{-2} s^{-1}. The germinated pollen grains were examined under a microscope after incubation for overnight.

2.9 Microscopy

Pollens from T2 generation were examined under an Axio Imager fluorescent microscope (Zeiss company) equipped with camera and software AxioVision. ApoTome from Carl Zeiss is generating optical sections by means of “structured illumination”. Fluorescence was excited with the X-Cite Series 120 fluorescence lamp (EXFO) and the sharp image information from the focal plane which is overlaid with blurred image

information from out-of-focus planes. *Arabidopsis* Pollen from wild type and transformed stp9.gfp, stp9.tp.gfp and stp9.tp.gfp.rfp.aap3 plants were spread on glass slide containing a water drop in the middle. Dic-filter was used for having pictures under normal illumination. While 38 HE GFP filter was used with GFP fusion and dsRED filter was used with RFP fusion.

3 Results

Most molecular approaches with potential for controlling gene flow among crops and weeds have thus far focused on maternal inheritance, male sterility, and seed sterility (Daniell, 2002). *Arabidopsis* exhibits Lycopersicon type of plastid inheritance, and transmits its plastids maternally (Nagata *et al.,* 1999). The maternal transmission of plastids in *Arabidopsis* is not absolute but there is some leakage of paternal plastids into subsequent generations via pollen exhibiting very low frequency 3.9×10^{-5}, and this leakage performed under a bias of manual pollination and selection on callus induction medium (Azhagiri and Maliga, 2007).

The study of organelle inheritance began with the use of phenotypic markers in genetic study like leaf pigment mutations that were readily apparent as variegated sectors (Cornu and Dulieu, 1988), plastome mutations including resistance to antibiotic selectable in culture (Avni and Edelman, 1991; Horlow *et al*., 1990; Ruf *et al*., 2007; Svab and Maliga, 2007; Azhagiri and Maliga, 2007), plastome mutations including resistance to herbicide that could be tested in the field (Wang *et al*., 2004). Plastid transmission is very likely controlled by nuclear genes (Azhagiri and Maliga, 2007), but to date, not a single gene involved in plastid inheritance has been identified in any higher plant. In other words, the molecular mechanism of plastid inheritance is still not clear.

3.1 Optimization of Atrazin Concentration

In order to set up a genetic screening approach, a selectable marker was chosen. In our case we chose atrazin resistance as a marker for plastid inheritance. Therefore the optimal dosage of atrazin application had to be determined. Preliminary experiment for optimizing the atrazin conditions required for genetic screening was

performed as following: Seeds from atrazin resistant plants (ElyF3BC4 & M2ElyF3BC4) besides seeds from atrazin sensitive wild type plants *(Ler*) were sterilized by gas sterilization and germinated on ms-plates containing different atrazin concentrations 0µm as a control, 5 µm, 10 µm, 25 µm and 50 µm atrazin. Plants containing atrazin-resistant plastid type had the ability to germinate on atrazin-containing ms medium while the plants containing atrazin-sensitive plastid type could not germinate as shown in Figure 5.

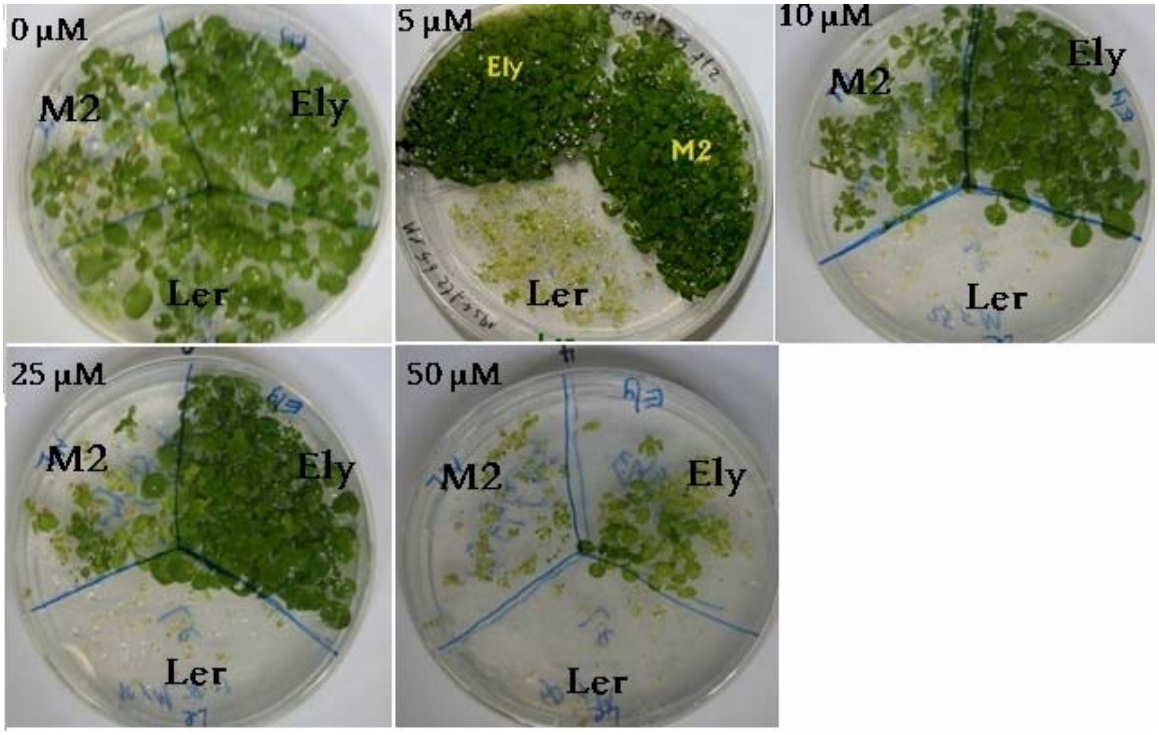

Figure 5. Three weeks old plants (Ler, ElyF3BC4 and M2ElyF3BC4) grown on ms-plates containing different atrazin concentrations (0, 5, 10, 25 and 50 µM).

There was no obvious difference in phenotypic growth parameters between atrazin resistant seedlings (ElyF3BC4 & M2ElyF3BC4) and wild type atrazin sensitive Ler plants

on ms medium without atrazin. On the other hand, the germination of atrazin resistant seedlings (ElyF3BC4 & M2ElyF3BC4) and wild type atrazin sensitive *Ler* plants were greately affected by high atrazin concentration 50 µM. We found 10 µM concentration of atrazin and 3-4 weeks growth period as the best condition for screening plastid-inherited atrazin resistance in *Arabidopsis* plants.

3.2 Screening of Paternal Plastids in *Arabidopsis* Hybrids generated by Spontaneous Out-crossing under Field Conditions

To quantify the bio-safety degree of paternal plastid inheritance, the frequency of paternal leakage of plastids via pollen under field conditions in *Arabidopsis thaliana* was estimated in out-crossing experiments (Figure 2) in collaboration with Rostock University Group (Prof. Broer) in 2008 and 2009. In the out-crossing experiment, the pollen donor atrazin-resistant plant ElyF3BC4 (male fertile) was grown beside the pollen recipient atrazin-sensitive male sterile N75 and atrazin-sensitive, male fertile wild type *Col-0*. Male sterile N75 plant can receive air-dispersed pollens from neighbor flowers. Two genetic out-crossing experiments (*Arabidopsis* field trials 2008 & *Arabidopsis* field trials 2009) including high pollen pressure and low pollen pressure out-crossing were set up. The pollen donor and pollen recipient plant types in high pollen pressure experiment were the same plant types in low pollen pressure experiment. In high pollen pressure trial, one row pollen recipient either N75 or *Col-0* plants in the middle of each parcel was surrounded by 6 rows of pollen donor ElyF3BC4 plants. While in the low pollen pressure trial, pollen recipient N75 or Col-0 plants were cultivated in an alternative way side by side, with pollen donor ElyF3BC4 plants (see chapter 2.5).

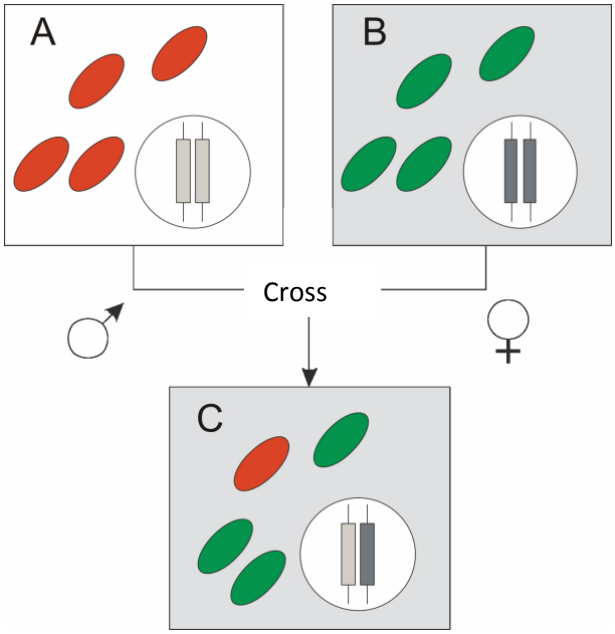

Figure 6. Out-crossing experiment. A: Pollen donor plant. Plastids are equipped with a marker (red); in this case atrazin resistance. B: Recipient plant, wild type line with unmodified plastids (green). C: Progeny of wild type line. A plastid (red) has been inherited via the pollen.

3.2.1 *Arabidopsis* Field Trials 2008

In field trials 2008, N75 and *Col-0* plants were the pollen recipient plants while ElyF3BC4 plants were the pollen donor plants in both the high-pollen-pressure experiment and the low-pollen-pressure experiment. Homozygous male sterile plants N75 could not produce pollen and subsequently no seeds after self-crossing, while

heterozygous N75 plants produced pollen. Self-pollination of heterozygous N75 resulted in 25% homozygous male sterile plant progeny, 50% heterozygous N75 plant progeny and 25% wild type plant progeny. Since both heterozygous N75 and wild type plants had the ability to produce pollen and seeds after self crossing, it was difficult to distinguish between them. For propagation of N75 plants, the cross N75 x *Ler* was done, then heterozygous plants were allowed to self. The whole seeds resulting from selfing hybrid N75 plants (F2 plants) were used as pollen recipient plants in the out-crossing experiment 2008. These F2 seeds collectively were used as mother plants without isolating the sterile N75 plants (homozygous N75) away from the fertile N75 plants (heterozygous N75).

3.2.1.1 Harvesting of *Arabidopsis* Seeds in Field Trials 2008

Seeds were harvested separately by the Broer group after ripening of siliques from all rows containing recipient plants N75 or *Col-0* in high and low pollen pressure trials. By roughly counting of seeds, we found that 1 ml volume seeds had a number of about 100,000 (10^5) seeds. Seeds were sterilized by gas sterilization method and screened on ms-medium containing 10 µM atrazin.

First we screened 1 ml seeds from each recipient (N75 and *Col-0*) plants in high-pollen-pressure and low-pollen-pressure trials besides positive and negative controls as in Table 1. Because seed contamination was found in the high-pollen-pressure experiment especially in that containing N75 as pollen recipient plant, we screened only the seeds produced from the high pollen pressure trial N75 x ElyF3BC4 (Table 1). Although we encountered 193 green seedlings displaying atrazin resistance from

4.850.000 total seeds screened on atrazin medium showing corresponding to a frequency 3.9 x10^{-5}.

Table 1. Number of seeds and candidates screened on 10 µM atrazin-containing ms-plates in *Arabidopsis* trials 2008.

Trials 2008	Crosses	Parcels and Rows	No. of seeds	No. of Candidates (Atrazin^{R} plants)	Candidates frequency
High Pollen Pressure (Trial 1)	N75 x ElyF3BC4	P1.1 R4	950,000	8	0.4 x10^{-5}
		P1.2 R4	1,350,000	5	
		P1.3 R4	1,650,000	5	
	Col.o x ElyF3BC4	P1.4 R4	100,000	57	36 x10^{-5}
		P1.5 R4	100,000	18	
		P1.6 R4	100,000	35	
Low Pollen Pressure (Trial 2)	N75 x ElyF3BC4	P2.1 R2	100,000	4	13.3 x10^{-5}
		P2.2 R2	100,000	28	
		P2.3 R2	100,000	8	
	Col.o x ElyF3BC4	P2.4 R2	100,000	11	8.3 x10^{-5}
		P2.5 R2	100,000	7	
		P2.6 R2	100,000	7	
Negative Control	N75 x Ler	P3.1 R4	many	0	
		P3.2 R4	many	0	
	Ler	P3.1 R1+7	many	0	
Positive Control	ElyF3BC4	P1.1 R4	many	all	
Total			4.850.000	193	3.9 x10^{-5}

3.2.1.2 Molecular analysis for *psbA* gene

A molecular marker for the *psbA* gene from wild type *Ler* and ElyF3BC4 was developed through PCR-RFLP analysis of plastid-DNA. First, total cellular DNA was extracted, then ~ 478 bp of *psbA* gene containing the mutation site domain was amplified from ptDNA by PCR using D1 primers forward and reverse. After BstXI digest (Figure 7) of the amplification products, distinct organelle DNA genotypes were recognized by exhibiting different restriction fragment patterns. Two bands (265 and 213 bp) were characteristic for the atrazin-resistant ElyF3BC4, while the band (478 bp) was found in atrazin-sensitive wild type. In the 193 candidates, only the ElyF3BC4 genotype could be detected although for hybrid plastids we would have expected a mixture of both genotypes.

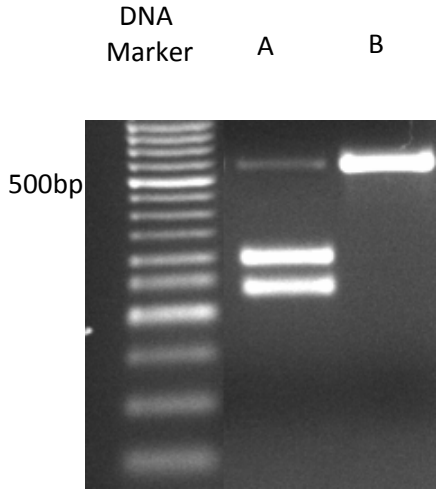

Figure 7. BstXI digest pattern in ElyF3BC4 (A) , and wild type *Ler* (B)

3.2.1.3 Molecular Analysis of Nuclear genes using Nuclear Markers

Nuclear genotyping in the candidates was performed by using the nuclear genetic markers NGA8 (chromosome 4) and F16j7TRB (chromosome 1) which distinguish between *Col-0* back-ground and ElyF3BC4, which is essentially *Ler*. This assay was used to genotype the 135 candidates resulting from the cross Col-0 x ElyF3BC4 (Table 3). Polymorphism analyses for the 135 resistant plants were performed by PCR using the forward and reverse primers of genetic markers NGA8 and F16j7TRB respectively. In all 135 atrazin resistant plants only ElyF3BC4 polymorphism pattern was recognized, indicating that the candidates did not result from paternal leakage.

3.2.1.4 Molecular analysis for *MS1* gene 2008

To discriminate hybrid nucleus of N75 and ElyF3BC4, the *MS1* gene was used as molecular marker. The *MS1* gene was amplified from ElyF3BC4and N75 and sequenced. The sequencing chromatogram for *MS1* gene (Figure 4) showed a G base at position 882 in ElyF3BC4 nuclear genome while this G base was changed to A base in N75 nuclear genome. In an artificial hybrid genome (DNA of N75 and ElyF3BC4 mixed) both G and A bases were detected at the same position 882. After sequencing the *MS1* gene isolated from genomic DNA of 58 candidates, we could not encounter any true hybrid sequence in the sequencing chromatogram.

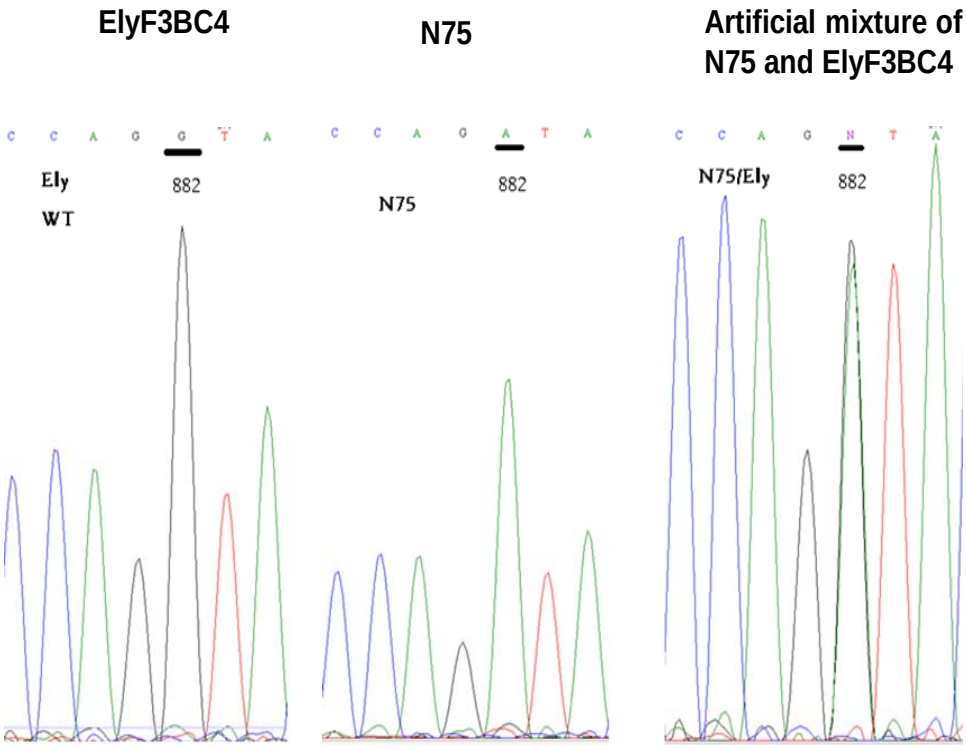

Figure 8. Sequencing chromatogram of flanking one base mutation of *MS1* gene in Ely (882 A), N75 (882 G) and hybrid N75/Ely (882 N). N means A+G

3.2.1.5 Out-crossing Rate under Field Conditions

To determine the out-crossing rate in the cross N75 x ElyF3BC4, 8 progeny plants were analyzed. In case of out-crossing, one expect the existence of both parental genotypes in the hybrid progeny while one genotype N75 or ElyF3BC4 was expected to exist after selfing. After spontaneous crossing, these 8 progeny plants were grown on

MS-medium without atrazin, to promote healthy growth. Afterwards DNA was extracted from 8 plants and subjected to *MS1* sequencing. In these 8 plants only 4 plants were found to be hybrids (Table 2).

Plants grow on MS-medium without Atrazin	Ms1.1 sequence	Results
1	agg	Ely F3BC4
2	agg	Ely F3BC4
3	agn	N75/ElyF3BC4
4	agn	N75/Ely F3BC4
5	agg	Ely F3BC4
6	agn	N75/Ely F3BC4
7	agn	N75/Ely F3BC4
8	aga	N75

Table 2. *MS1* gene sequencing data quantifying outcrossing rate among male sterile plants (N75) and ElyF3BC4 plants screened on ms-plates free from atrazin. In this case agg= ElyF3BC4 or Ler, aga = N75 and agn= hybrid , n=g+a.

To summarize the results from field trial 2008 (Table 2 and Table 3): about 50% of the seeds produced from the cross N75 x ElyF3BC4 resulted from out-crossing and the other 50% produced from self-crossing of N75 or ElyF3BC4 plants. Therefore, the frequency was modified from 3.9×10^{-5} to 1.98×10^{-5}. After molecular analysis of plastid gene markers (in *psbA* gene) and nuclear markers (in *MS1* gene and NGA8 & F16j7TRB), we found 193 atrazin-resistant candidates. All of them exhibited only

ElyF3BC4 genome back-ground in the plastid and nucleus representing seed contamination.

Table 3. PCR-RFLP analysis of Plastid DNA marker for *psbA* gene and nuclear markers NGA8, F16J7TRB besides marker in *MS1* gene used to distinguish between false and true candidates in out-crossing experiments 2008.

Trials 2008	Crosses	Parcels and Rows	PCR-RFLP Analysis	*MS1* Sequencing	NGA8	F16J7 TRB
High Pollen Pressure (Trial 1)	N75 x ElyF3BC4	P1.1 R4	ElyF3BC4	ElyF3BC4	-----	-----
		P1.2 R4	ElyF3BC4	ElyF3BC4	-----	-----
		P1.3 R4	ElyF3BC4	ElyF3BC4	-----	-----
	Col.0 x ElyF3BC4	P1.4 R4	ElyF3BC4	-----	ElyF3BC4	ElyF3BC4
		P1.5 R4	ElyF3BC4	-----	ElyF3BC4	ElyF3BC4
		P1.6 R4	ElyF3BC4	-----	ElyF3BC4	ElyF3BC4
Low Pollen Pressure (Trial 2)	N75 x ElyF3BC4	P2.1 R2	ElyF3BC4	ElyF3BC4	-----	-----
		P2.2 R2	ElyF3BC4	ElyF3BC4	-----	-----
		P2.3 R2	ElyF3BC4	ElyF3BC4	-----	-----
	Col.o x ElyF3BC4	P2.4 R2	ElyF3BC4	-----	ElyF3BC4	ElyF3BC4
		P2.5 R2	ElyF3BC4	-----	ElyF3BC4	ElyF3BC4
		P2.6 R2	ElyF3BC4	-----	ElyF3BC4	ElyF3BC4
Negative Control (Trial 3)	N75 x Ler	P3.1 R4	-----	-----	-----	-----
		P3.2 R4	-----	-----	-----	-----
	Ler	P3.1 R1+7	ler	-----	-----	
+ve Control	ElyF3BC4	P1.1 R4	ElyF3BC4	-----	-----	-----

3.2.2 *Arabidopsis* Field Trials 2009

A spontaneous out-crossing experiment, in which male sterile N75 plants were the pollen recipient and male fertile ElyF3BC4 plants were the pollen donor, was designed under field conditions in 2009. The set up of *Arabidopsis* field trials in 2009 was essentially the same as the set up in *Arabidopsis* field trials in 2008, except for two changes: first *Col-0* plants were excluded from cross breeding in low and high pollen pressure trials, second fertile plant lines and sterile plant lines from N75 were harvested separately. Exclusion of Col-0 plants from *Arabidopsis* field trials in 2009 enhanced the capacity to have hybrid plants, because *Col-0* plants have a strong tendency towards self-pollination. Another advantage came from using only sterile lines from N75 plants as pollen recipient and exclusion of fertile lines.

3.2.2.1 Harvesting of *Arabidopsis* Seeds in the Field Trial 2009

Ripened seeds from recipient plants N75 in high and low pollen pressure trials were harvested, sterilized by gas sterilization method and then screened on 10 µM atrazin containing ms-medium. The number of screened seeds and number of candidates on atrazin medium are illustrated in Table 4.

In general, we encountered 83 green candidate seedlings from 19.465 total seeds screened on atrazin medium (426 $x10^{-5}$).

Table 4. Number of seeds and candidates screened on 10 µM atrazin-containing ms-plates in *Arabidopsis* trials 2009.

Trials 2009	Crosses	Parcels and Rows	No. of seeds	No. of Candidates (Atrazin^R plants)	Candidate frequency
High Pollen Pressure (Trial 1)	N75 x ElyF3BC4	P1.1 R4	850	4	642 x10^{-5}
		P1.2 R4	1700	13	
		P1.3 R4	1000	6	
		P1.4 R4	1600	8	
		P1.5 R4	450	5	
Low Pollen Pressure (Trial 2)	N75 x Ely	P2.1 R2	1700	3	338 x10^{-5}
		P2.3 R2	2600	15	
		P2.4 R2	3900	9	
		P2.5 R2	1715	2	
		P2.6 R2	3950	18	
Negative Control	N75 x Ler	P3.1 R4	many	0	
		P3.2 R4	many	0	
Positive Control	Ely	P1.1 R4	many	all	
Total			19.465	83	426 x10^{-5}

3.2.2.2 Molecular analysis for *psbA* gene

PCR-RFLP analysis of the *psbA* gene in 83 candidates was performed. In these 83 candidates (Table 5) only the ElyF3BC4 genotype could be detected.

3.2.2.3 Molecular analysis for *MS1* gene

True instances of paternal leakage should result in hybrid nucleus containing hybrid alleles of the *MS1* gene. The sequencing chromatogram for *MS1* gene showed a G base at position 882 in all 83 candidates.

Table 5. PCR-RFLP analysis of Plastid DNA marker in *psbA* gene and sequencing of nuclear marker in *MS1* gene used to distinguish between N75 and ElyF3BC4 in out-crossing experiment 2009.

Trials 2009	Crosses	Parcels and Rows	PCR-RFLP analysis	*MS1* Sequencing
High Pollen Pressure (Trial 1)	N75 x ElyF3BC4	P1.1 R4	ElyF3BC4	ElyF3BC4
		P1.2 R4	ElyF3BC4	ElyF3BC4
		P1.3 R4	ElyF3BC4	ElyF3BC4
		P1.4 R4	ElyF3BC4	ElyF3BC4
		P1.5 R4	ElyF3BC4	ElyF3BC4
Low Pollen Pressure (Trial 2)	N75 x ElyF3BC4	P2.1 R2	ElyF3BC4	ElyF3BC4
		P2.2 R2	ElyF3BC4	ElyF3BC4
		P2.3 R2	ElyF3BC4	ElyF3BC4
		P2.4 R2	ElyF3BC4	ElyF3BC4
		P2.5 R2	ElyF3BC4	ElyF3BC4
		P2.6 R2	ElyF3BC4	ElyF3BC4
Negative Control	N75 x Ler	P3.1 R4	Wt	-----
		P3.2 R4	Wt	-----
Positive Control	ElyF3BC4	P1.1 R4	ElyF3BC4	-----

We did not encounter any hybrid sequence in the sequencing chromatogram. Therefore, 83 candidates that recognized on atrazin medium and showed ElyF3BC4 DNA background in plastid by PCR-RFLP analysis of *psbA* gene represent seed contamination.

3.3 Screening of Paternal Plastids in *Arabidopsis* Hybrids generated by manual Crossing

To search for a mutant plant exhibiting enhanced ability for plastid transmission via pollen, manual pollination of *Ler* with M2ElyF3BC4 pollen was performed.

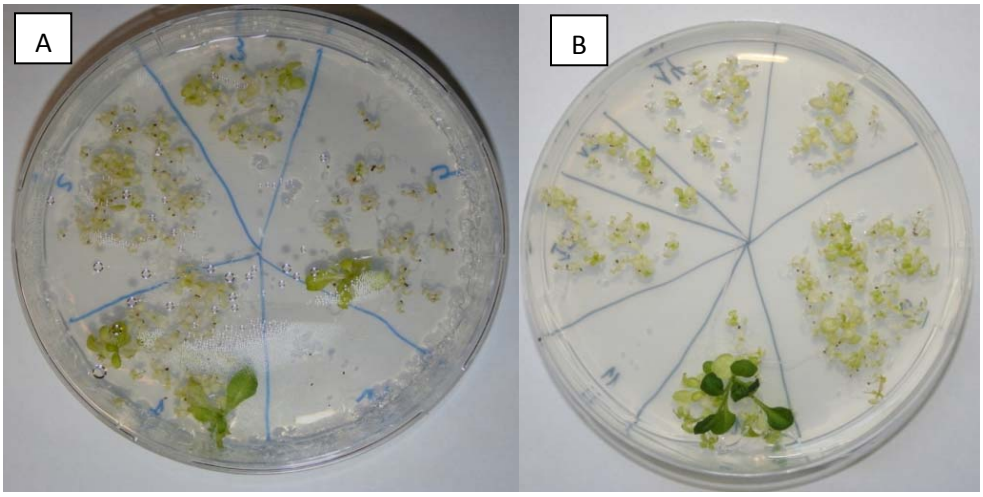

Figure 9. Two candidates 13.45 (A) and 19.2 (B) screened on ms-medium containing 10 µM atrazin after manual pollination of Ler stigma by M2ElyF3BC4 pollen under green house conditions.

In green house, pollens from M2ElyF3BC4 flowers were transferred manually by special crossing forceps to stigma of *Ler* gynoecium. Average number of seeds in ripened

siliques produced from each cross is 30 hybrid seeds. From 2178 successful crosses performed in the green house we obtained nearly 65,000 hybrid seeds. Hybrid seeds were collected separately and carefully to avoid seed contamination, then purified from non-seed materials. Afterwards they were sterilized by HCl gas under a hood. The hybrid seeds were selected on ms-medium containing 10 µM atrazin. After 3 weeks on ms + atrazin medium, we found two candidates 13.45 and 19.2 (Figure 9) corresponding to a frequency (3×10^{-5}).

3.3.1 Molecular Analysis of *psbA* gene

Molecular analysis of *psbA* gene in manual out-crossing experiment was achieved by PCR-RFLP analysis of *psbA* gene in plastid DNA. Two candidates were analyzed by the same method mentioned in natural out-cossing experiments (chapter 3.2.1.2 and chapter 3.2.2.2). The first candidate 13.45 showed no atrazin resistant PCR-RFLP pattern, while the second one 19.2 showed atrazin resistant RFLP pattern candidates. But we could not prove the hybrid nucleus existence in this candidate, because M2ElyF3BC4 and *Ler* have the same nuclear back-ground. We aimed to reproduce this phenotype from the respective M2ElyF3BC4 line (19.2). 54 progeny of this line were sown out and the crossing repeated as mentioned above. About 1600 seeds were screened on ms-medium containing 10 µM atrazin. In none of these seeds an atrazin resistant plant survived. This implies that the original survival of progeny seeds on atrazin medium might not be caused by paternal leakage.

3.4 Genetic Analysis of Plastid Inheritance through Autophagy ATG4s Genes

Following the hypothesis that plastids might be degraded in generative or sperm cells in Arabidopsis pollen under the control of autophagy genes, genetic analysis experiment were designed to test if the nuclear autophagy ATG4s (ATG4a and 4b) genes are involved in maternal inheritance of plastids in *Arabidopsis*. Double mutant *atg4a4b-1* plants are defective in autophagy and there were no autophagic bodies in the vacuoles of *atg4a4b-1* plants (Yoshimoto *et al.,* 2004).

Therefore, we generated a mutant that combines the defect in the autophagy genes *ATG4a4b* present in (*atg4a4b-1* plant) and the plastid marker in *psbA* gene present in ElyF3BC4. To this end we performed the cross *ElyF3BC4* X *atg4a4b-1*, in which *atg4a4b*-1 mutant was the pollen donor. Homozygous *ElyF3BC4-atg4a4b* plants were obtained by transferring the pollens from double autophagy mutant *atg4a4b-1* to *ElyF3BC4* stigma. All F1 seeds grown normally on atrazin medium, 8 Hybrid plants were selected by PCR using the following primers atg4a-s, atg4a-as, atg4b-s, atg4b-as, pgap-1 and pgap-3. Afterwards the F2 generation was produced by self-pollination, 6 plants from 48 in F2 plants were homozygous for atg4a4b-1.

To test if *ElyF3BC4-atg4a4b-1* plants were able to transfer their plastids via pollen, 5 wild type *Ler* plants (5 flowers per each) were pollinated by 5 different mutant *ElyF3BC4-atg4a4b-1* plants manually. Nearly 750 F1 seeds were sterilized and screened on atrazin medium, but we did not encounter any resistant plant.

3.5 Visualization of Pollen Plastids via GFP-Fusion

Plastids in *Arabidopsis* pollen are not visible under the normal light microscope, because of the absence of chlorophyll (Tang *et al.,* 2009). To visualize plastids in pollen

grains, a *STP9.GFP* construct for labeling pollen cytoplasm and *STP9.TP.GFP* construct for labeling pollen plastids were created. First, cytoplasm in pollen was visualized by expression of green fluorescence protein GFP from the nuclear genome in pollen cytoplasm under the control of pollen promoter from *STP9* gene. In order to guide GFP

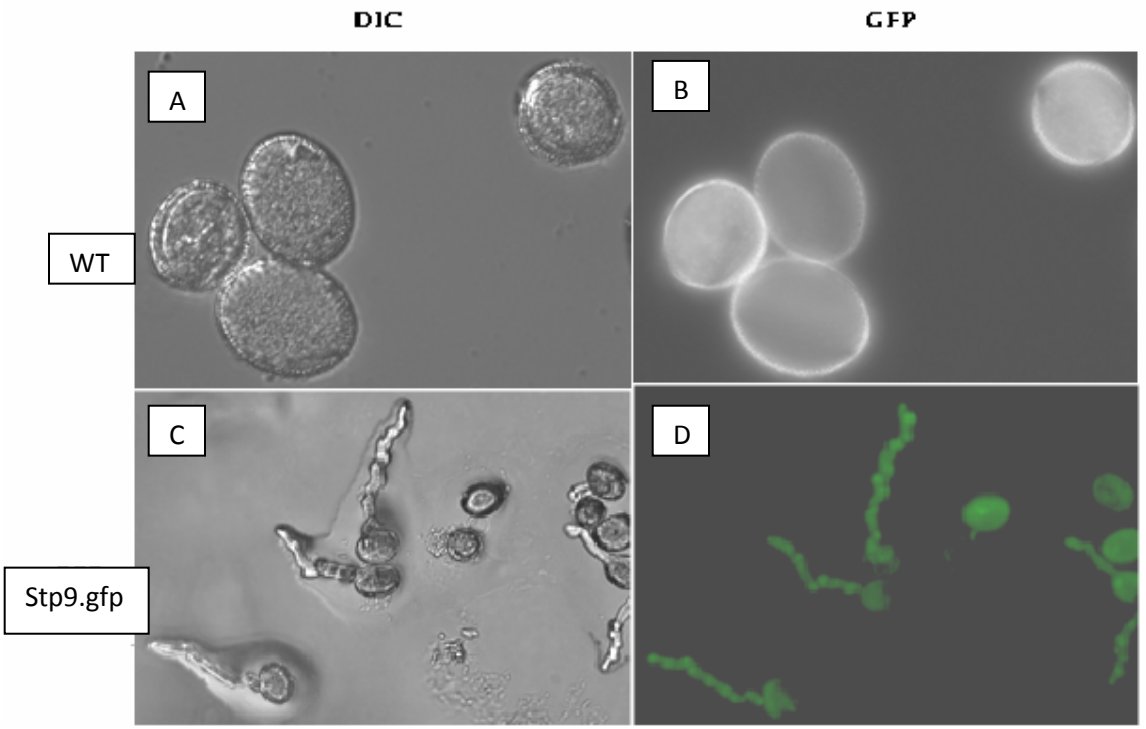

Figure 10. Fluorescent images of the pollen Stp9.gfp. Light microscopic image of a wild type pollen grain under Dic filter (A) and under GFP filter (B). tp.gfp transformed pollens under Dic filter (C) and under GFP filter (D).

to the plastids a transit peptide encoded by the first 300 bp from *GPT1* gene, was fused at the N-terminus. 10 *Arabidopsis* Col-0 plants for each construct were transformed with the *STP9.GFP* and *STP9.TP.GFP* constructs using the floral dipping technique. After transformation seeds were harvested from transformed lines separately. Then

thousands of T1 seeds were sterilized and screened on ms-medium containing kanamycin antibiotic. After selection, 15 transgenic plants (kanamycin resistant lines) from each construct were confirmed by PCR. The transgenic lines were grown on soil and selfed afterwards. The whole T2 seeds from one line were screened by the same way. 5 opened flowers from each construct were used for fluorescence microscopy. Fluorescence microscopy of pollens prepared from wild type Col-0, *stp9.gfp* plants and *stp9.tp.gfp* plants (Figure 10 & 11) showed that GFP signals in the whole pollen cytoplasm of *stp9.gfp* plants while GFP signals precisely arising from pollen plastids of *stp9.tp.gfp* plants.

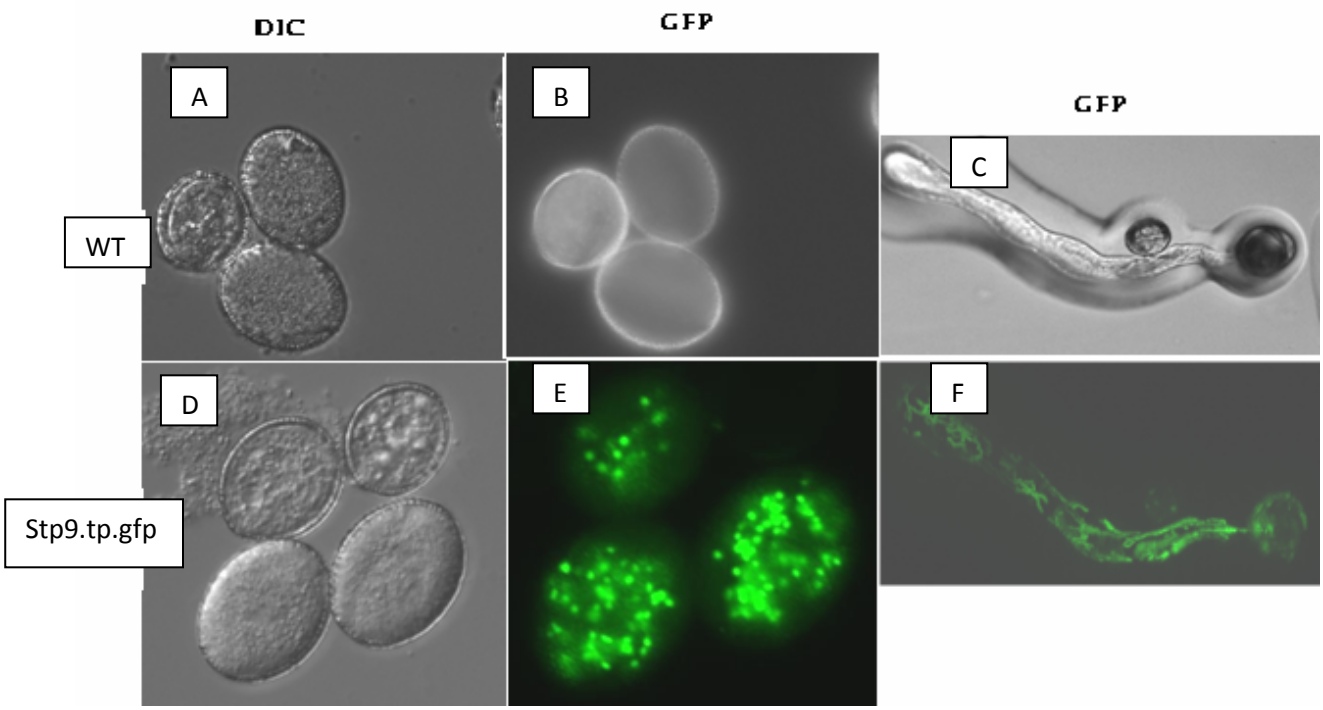

Figure 11. Fluorescent images of the pollen Stp9.tp.gfp. Light microscopic image of a wild type pollen grain under Dic filter (A) and under GFP filter (B). stp9.tp.gfp transformed pollens under Dic filter (D) and under GFP filter (E).Germinated pollens under GFP filter in wild type (C) and stp9.tp.gfp (F).

To differentiate between plastids existing in vegetative and generative cells in *Arabidopsis*, the *STP9.RFP.AAP3* construct was created. In this construct, the reporter gene RFP was expressed under control of the pollen promoter STP9. AAP3 is a plasma membrane transporter that is also present on internal membranes along the trafficking pathway (Okumoto *et al*., 2004). Using the floral dipping method, 10 *Arabidopsis stp9.tp.gfp* and 10 Col-0 plants were transformed with the *STP9.RFP.AAP3* construct. T1 seeds were sterilized and screened on ms-medium containing BASTA herbicide. After selection 15 transgenic plants (BASTA resistant lines) from each construct, they were confirmed by PCR. The transgenic lines were grown on soil and selfed afterwards. The whole T2 seeds from one line were screened on soil by BASTA spray. 5 opened flowers from *stp9.tp.gfp.rfp.aap3* and *stp9.rfp.aap3* transgenic plant were used for fluorescence microscopy. Fluorescence microscopy of pollens prepared from *stp9.rfp.aap3* and *stp9.tp.gfp.rfp.aap3* plants showed very weak RFP signal. In conclusion, colorless plastids in *Arabidopsis* pollen were visualized by the expressed GFP protein under the control of pollen promoter but the specification of plastids in vegetative and generative cells has to be improved.

4. Discussion

The aim of this study was to assess the frequency of plastid transfer from atrazin-resistant *ElyF3BC4 Arabidopsis thaliana* plants bearing a point mutation in the plastid *psbA* gene to male sterile N75 plants by spontaneous crossing under field conditions. Also the plastid transfer from atrazin-resistant, EMS-mutagenized *M2ElyF3BC4* plants to wild type *A. thaliana* plants by manual crossings under green house conditions was estimated. It was found that plastid-encoded atrazin resistance could not be transmitted via pollen, neither by manual pollination among 65,000 hybrid seeds nor by spontaneous pollination among 2,444,465 hybrid seeds in *A. thaliana*.

In a study by Maliga and coworkers, a rare exception to maternal inheritance was observed as leakage of paternal plastids at low 3.9 $X10^{-5}$ frequency to the next generation through pollen in *Arabidopsis* (Azhagiri and Maliga, 2007). This low frequency of paternal plastid transmission involved probably very few ptDNA copies, and could only be detected due to the screening procedure because sensitive tissue culture selection was employed to test for spectinomycin resistance encoded in the paternal ptDNA.

In our study, the herbicide atrazin, a photosystem II inhibitor was used as a selection material. It blocks the electron transport chain in chloroplast, resulting in the generation of active oxygen species. Atrazin also reduces the leaf pigment levels, especially chlorophyll a and decreases photosynthetic performance (Ivanov, 2005). In unpublished work from Sabine Jarzombski and Anja Schneider, the selection procedures on callus induction medium did not work, because photosynthetic *psbA* gene might be repressed or down regulated with other photosynthetic genes, when high sugar (5%)

content was used. Therefore, the amount of down regulated photosynthetic proteins including D1 protein might not be sufficient to perform the atrazin screening. On the other hand, 5 % sucrose was necessary to sustain the callus growth. In our work, atrazin selection of seedlings on ms medium might be not sensitive enough because if only a few paternal plastids were transferred to the hybrid progeny, it could be that photosynthetic products of the mutated atrazin-resistant are not sufficient for plant survive. Also the oxidation of proteins and lipids by active oxygen species besides the maternal inheritance are another factors playing a role in the observed zero frequency in this study. In our study, the frequency of zero of plastid transfer that we encountered may be because rare ptDNA copies might have remained below the detection limit. It was found that PCR-RFLP analysis could not detect the *ElyF3BC4* genome background below 10% in a synthetic mixture of *ElyF3BC4* and *Ler* DNA (unpublished work from Sabine Jarzombski and Anja Schneider).

Zero frequency gene flow from plastids is the rule in species exhibiting strict maternal inheritance and the low frequency gene flow from plastids is an exception to this rule. This zero frequency may be due to unknown genetic paternal control mechanism excluding plastids from pollen and genetic maternal control mechanism excluding paternal plastids during zygote or embryo formation. Therefore, the low frequency gene flow from plastids might be due to the relaxation in these two genetic (paternal and maternal control) mechanisms.

Our finding that various random nuclear mutations by EMS could not create a mutant able to transfer its paternal plastids into egg-cell of recipient plant might be explained by duplication or redundancy of nuclear genes in *Arabidopsis*. Thus a defect in one gene is compensated by the function of its homologue. In *Arabidopsis* about 1/6 of

the genes are segmentally duplicated, therefore redundancy can be observed frequently. To test this idea, nuclear defective in autophagy ATG4a and ATG4b genes of double mutant *atg4a4b-1* plant were employed as pollen donor plant. Autophagy has a relation with degradation of paternal plastids, where the temporary occurrence of small autophagic vacuoles inside the generative cell seems to be common in those species where plastid inheritance is maternal. The generative cell cytoplasm recognizes plastids, and eliminates them by means of autophagic vacuoles (Pacini *et al*., 1992). To the end, it was observed that also in the autophagy *atg4a4b-1* mutant no paternal plastid transfer occurred.

In conclusion, maternal inheritance of plastids in *Arabidopsis* minimizes the escape of plastid transgenes and fortifies the prevention of gene flow through pollen in plants having modified plastids.

4.1 Chloroplast Genetic Engineering

Plastid transformation in *Arabidopsis* is feasible but inefficient due to unknown reasons. *Arabidopsis* recorded 1% plastid transformation, but none of the plants regenerated from the transformed lines were fertile (Sikdar *et al*., 1998). Therefore, a natural herbicide resistant mutant ElyF3BC4, bearing a point mutation in the plastid *psbA* gene, was used by us to follow the plastid transmission through pollen from generation to generation in this study. On the other hand, tobacco remains the most amenable species to chloroplast genetic engineering to date. Also, plastid transformation has been achieved successfully in crops such as maize, tomato, cotton, potato, rice and sugar beets.

Chloroplast genetic engineering is a strategy consisting of inserting a transgene into the chloroplast genome of a plant instead of its nuclear genome. A single plastid gene is represented up to 10 000 times within a photosynthetically active cell. Plastid transformation has some advantages compared to nuclear transformation, including control of the site of gene insertion (by homologous recombination), high rates of transgene expression and protein accumulation and absence of epigenetic effects. Foreign proteins have been shown to accumulate to a level as high as 46% of the total leaf soluble protein (De Cosa *et al*., 2001), and the accumulation of transcripts is 169 times higher in chloroplasts than in plants after nuclear transformation (Lee *et al*., 2003).

Chloroplast genetic engineering technology is a promising tool providing less expensive drugs and vaccines by molecular pharming. Molecular pharming is the production of a wide range of products such as vaccine antigens, pharmaceutical proteins and industrial proteins, in economical way superior to bacterial cultivation or animal cell cultures, because plants do not require industrial bioreactors to produce a high mass harvest. Since the therapeutic compound is produced and stored in plant tissue, it might be possible to apply these compounds simply by eating the plant without isolating the desired pharmaceutical. Another aspect of chloroplast engineering is to improve agricultural traits such as herbicide and pathogen resistance, resistance to drought, salt tolerance and phyto-remediation (Řepková, 2010).

For the delivery of the recombinant DNA plasmid into plastids, either particle bombardment-mediated transformation (gene gun) or polyethylene glycol (PEG) treatment of leaf protoplast in the presence of plasmid DNA were used (Golds *et al.,* 1993). In homologous recombination, the flanking sequences guide the recombinant

DNA into a specific place on the chloroplast genome by binding to corresponding parts on the genome. The protoplasts are then grown on a plate containing an antibiotic, as selectable marker. These surviving cells are then exposed to regenerative factors that induce shoots and grow into full plants that express the desired protein (Sikdar, 2002). Any undesired sequences integrated by chance into the nuclear genome are easily removed by backcrossing the transgenic plants with an untransformed plants used as pollen parent.

4.2 Nuclear Gene Flow from Gene-modified Plants to Non-gene Modified Plants

Additionally, nuclear transformation of plants is not very efficient because there is only one nucleus per cell and, at most, a few copies of the recombinant gene, producing relatively low levels of protein. Transforming the nuclear DNA plants is one of the most discussed topics in our days (the GMO discussion = genetically modified organisms).

If recombinant genes were to be disseminated through pollen and integrated into other plants, invasive species and widespread ecological damage could result. For example, nuclear transgenes seemed to escape to from gene modified (GM) plants to non-gene modified plants in Europe a few years ago when non-GM products with 0.9% contamination of GM products were marketed without labeling. Gene flow in nuclear transformed plants can be high if these GM seeds germinate, grow, flower at the same time, and be close enough in space to allow for the transfer of pollen, there is a risk that interbreeding with a sexually compatible weedy species could produce a fertile hybrid (Daniell, 2007).

Nuclear transformation might be harmful to the plant itself because the transgenes are integrated into the plant's nucleus at random positions.

4.3 Plastid Gene Flow from Gene-modified Plants to Non-gene Modified Plants

Chloroplasts are an ideal place to accumulate proteins or their biosynthetic products that may be harmful in the cytoplasm, such as cholera toxin B subunit, trehalose or xylanase (Bogorad, 2000).

Plastid transformation method ensures that the recombinant transgenes are contained within the chloroplast and their effects can be more easily controlled. Plastid genes will not spread to other plants because they are not passed into the sperm cells of plant pollen, so plastids cannot be spread by pollination. In conclusion, plastid transformation technique is environmentally friendly.

4.4 Maternal Inheritance as Transgene Confinement Tool

A recent report from the European Environment Agency (Copenhagen, Denmark) recommends chloroplast genetic engineering as a gene-containment approach (Eastham and Sweet, 2002). This recommendation was based on the results of gene flow studies and biosafty measurements. The transgene escape from transplastomic crops is rare and poses a negligible risk if plants are genetically engineered via the chloroplast genome. Other researchers prefer to reduce the risk of plastid gene flow to zero for a subset of applications (Daniell, 2002; Lee and Natesan, 2006). To have zero plastid gene flow, most molecular approaches with potential for controlling gene flow

among crops and weeds have thus far focused on maternal inheritance and male sterility (Daniell, 2002). As in this study, it is difficult to control nuclear gene flow, but plastid gene flow at least in *Arabidopsis* recorded a very low 3.9×10^{-5} frequency (Azhagiri and Maliga, 2007) or zero frequency in this study.

It needs more experiments to compare the transgene containment level in field through spontaneous pollination and the transgene containment level in greenhouse through manual pollination.

4.5 Mechanism of Plastid inheritance

Very little is known about how plastid DNA (ptDNA) is distributed into daughter plastids upon plastid division. Plastid DNAs in higher plants do not normally undergo sexual recombination, even when they are inherited biparentally. This means that, chloroplast fusion and genetic recombination does not occur in higher plants (Baldev *et al*. 1998).

Diversity in the mode of organelle inheritance within families (Hansen et al., 2007; Havey et al., 1998) and in the frequency of paternal exceptions within species (Derepas and Dulieu, 1992), suggest that plastid transmission is very likely controlled by nuclear genes (Azhagiri and Maliga, 2007), but to date, not a single gene involved in plastid inheritance has been identified in any higher plant.

From the contribution ratio of paternal and maternal plastids in plants which transmit their plastids biparentally or maternally, there are gradients of these plastid contribution ratios even in plants showing the same type of plastid inheritance. It is

suggested that organelle inheritance is a quantitative trait. For example, although *Medicago* and *Pelargonium* are transmitting their plastids by the same way (maternaly), but the paternal plastid contribution in *Medicago* plant is much greater than the paternal plastid contribution in *Pelargonium*. Also in *Chlorophytum comosum*, a species known to display biparental inheritance, paternal plastids are inherited at a rate of 2–8% (Pandey and Blaydes 1957). So possession of biparental inheritance does not mean an equal proportion of plastids are received from the two parents. Nor does maternal inheritance mean that plastid transmission from the male parent never occurs. For instance, in *Nicotiana tabacum*, a representative of species with maternal inheritance, the frequency of paternal plastid transmission was reported in the range from 10^{-4} to 10^{-5} (Ruf *et al*., 2007; Svab and Maliga, 2007) up to 2.5% of the seed progeny (Avni and Edelman, 1991).

However, it is clear that plastids are excluded from the male gametic cells of angiosperms displaying maternal plastid inheritance (Hagemann and Schroeder 1989). Therefore, a common cytological tag for maternal inheritance is that the generative or sperm cells are free from plastids, and accordingly lack plastid DNA. Although the cytological investigations for classification of plastid inheritance are based on DAPI staining, DAPI signals do not represent the quantity of DNAs. They tend to show the physiological state in which ptDNA is condensed with associating proteins. Likewise, the absence of DAPI signals does not mean that organelle DNAs are completely missing, as evidenced by several exceptional species showing biparental inheritance. Nevertheless, it should be noted that the ptDNA signals detected by DAPI are missing even in the vegetative cells which are unlike the sperm cells and do not contribute to fertilization (Corriveau, 1991).

This study aimed to follow the behavior of plastids in vegetative and generative or sperm cells by direct visualization. GFP-labelled plastids in Arabidopsis pollen is effective method for direct observation of plastids. The GFP-fusion protein, however, is a nuclear localized protein but controlled by a pollen-specific promoter. An N-terminally fused transit peptide directs the GFP to plastids. Fluorescence microscopy of *stp9.tp.gfp* pollens showed a strong signals arising from plastids, but could not distinguish between plastid localized in vegetative cells and plastids localized in generative or sperm cells. This non–defined localization comes from invisible plasma membrane especially the membrane enclosing the generative or sperm cells. Since there are no specific dyes for plasma membrane, we tried to label the plasma membrane by using a RFP-Fusion protein. It turned out that the dsRFP signals were very weak. For detection these signals could be so weak because the expressed proteins may be unstable or rapidly degraded.

4.6 Outlook

In the present study, the plastid inheritance was followed by using herbicide marker which may not be ideal or not sensitive enough to carry out such study. A more sensitive screening system might be more successful. For example, investigation of plastid inheritance could be achieved by using GFP marker in *stp9.tp.gfp* mutant as selectable marker, if the expression of GFP gene is limited to pollen. This line could be mutagenized via EMS mutagenesis. These mutant plants could then be used as pollen donor to fertilize wild-type egg-cells. After fertilization the fertilized egg-cells could be screened for GFP labeled plastids.

References

Aldridge, C., Maple, J. and Moller, S.G. (2005) The molecular biology of plastid division in higher plants. *Journal of Experimental Botany*, 56: 1061-1077.

Avni, A. and Edelman, M. (1991) Direct Selection for Paternal Inheritance of Chloroplasts in Sexual Progeny of Nicotiana. *Molecular & General Genetics*, 225: 273-277.

Azhagiri, A.K. and Maliga, P. (2007) Exceptional paternal inheritance of plastids in Arabidopsis suggests that low-frequency leakage of plastids via pollen may be universal in plants. *Plant J*, 52: 817-823.

Baldev, A., Gaikwad, K., Kirti, P.B., Mohapatra, T., Prakash, S. and Chopra, V.L. (1998) Recombination between chloroplast genomes of Trachystoma ballii and Brassica juncea following protoplast fusion. *Molecular and General Genetics*, 260: 357-361.

Bassham, D.C., Laporte, M., Marty, F., Moriyasu, Y., Ohsumi, Y., Olsen, L.J. and Yoshimoto, K. (2006) Autophagy in development and stress responses of plants. *Autophagy*, 2: 2-11.

Baur, E. (1909) Das Wesen und die Erblichkeitsverhältnisse der ‘‘ Varietates albomarginatae hort. ’’ von *Pelargonium zonale.* Z Indukt Abstammungs- Vererbungs 1: 330-351.

Bendich, A.J. (1987) Why do chloroplasts and mitochondria contain so many copies of their genome? *Bioessays*, 6: 279-282.

Birky, C.W., Jr. (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc Natl Acad Sci U S A*, 92: 11331-11338.

Boavida, L.C., Becker, J.D. and Feijo, J.A. (2005a) The making of gametes in higher plants. *International Journal of Developmental Biology*, 49: 595-614.

Boavida, L.C., Vieira, A.M., Becker, J.D. and Feijo, J.A. (2005b) Gametophyte interaction and sexual reproduction: how plants make a zygote. *International Journal of Developmental Biology*, 49: 615-632.

Bogorad, L. (2000) Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions and products. *Trends Biotechnol*, 18: 257-263.

Clauss, M.J. and Koch, M.A. (2006) Poorly known relatives of Arabidopsis thaliana. *Trends Plant Sci*, 11: 449-459.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J*, 16: 735-743.

Cornu, A. and Dulieu, H. (1988) Pollen Transmission of Plastid-DNA under Genotypic Control in Petunia-Hybrida Hort. *Journal of Heredity*, 79: 40-44.

Correns, C. (1909) Vererbungsversuche mit bla (gelb) grünen und buntblättrigen Sippen bei *Mirabilis jalapa Urtica pilulifera* und *Lunaria annua*. Z Indukt Abstammungs - Vererbungs1: 330-351

Corriveau, J.L. and Coleman, A.W. (1991) Monitoring by epifluorescence microscopy of organelle DNA fate during pollen development in five angiosperm species. *Dev Biol*, 147: 271-280.

Daniell, H. (2002) Molecular strategies for gene containment in transgenic crops. *Nat Biotechnol*, 20: 581-586.

Daniell, H. (2007) Transgene containment by maternal inheritance: effective or elusive? *Proc Natl Acad Sci U S A*, 104: 6879-6880.

De Cosa, B., Moar, W., Lee, S.B., Miller, M. and Daniell, H. (2001) Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nat Biotechnol*, 19: 71-74.

Derepas, A. and Dulieu, H. (1992) Inheritance of the Capacity to Transfer Plastids by the Pollen Parent in Petunia-Hybrida Hort. *Journal of Heredity*, 83: 6-10.

Diers, L. (1967) [Transmission of plastids by pollen in Antirrhinum majus]. *Mol Gen Genet*, 100: 56-62.

Eastham, K., Sweet, J., European Environment Agency. and European Science Foundation. (2002) *Genetically modified organisms (GMOs) : the significance of gene flow through pollen transfer : a review and interpretation of published literature and recent/current research from the ESF 'Assessing the Impact of GM Plants' (ASIGM) programme for the European Science Fountain and the European Environmental Agency / authors, Katie Eastham and Jeremy Sweet* Copenhagen, Denmark: European Environment Agency.

El-Lithy, M.E., Rodrigues, G.C., van Rensen, J.J., Snel, J.F., Dassen, H.J., Koornneef, M., Jansen, M.A., Aarts, M.G. and Vreugdenhil, D. (2005) Altered photosynthetic performance of a natural Arabidopsis accession is associated with atrazine resistance. *J Exp Bot*, 56: 1625-1634.

Fojtova, M., Kovarik, A. and Matyasek, R. (2001) Cytosine methylation of plastid genome in higher plants. Fact or artefact? *Plant Sci*, 160: 585-593.

Fujie, M., Kuroiwa, H., Kawano, S., Mutoh, S. and Kuroiwa, T. (1994) Behavior of Organelles and Their Nucleoids in the Shoot Apical Meristem during Leaf Development in Arabidopsis-Thaliana L. *Planta*, 194: 395-405.

Gastony, G.J. and Yatskievych, G. (1992) Maternal Inheritance of the Chloroplast and Mitochondrial Genomes in Cheilanthoid Ferns. *American Journal of Botany*, 79: 716-722.

Golds, T., Maliga, P. and Koop, H.U. (1993) Stable Plastid Transformation in Peg-Treated Protoplasts of Nicotiana-Tabacum. *Bio-Technology*, 11: 95-97.

Guillon, J.M. and Raquin, C. (2000) Maternal inheritance of chloroplasts in the horsetail Equisetum variegatum (Schleich.). *Current Genetics*, 37: 53-56.

Hagemann, R. (2000) Erwin Baur or Carl Correns: who really created the theory of plastid inheritance? *J Hered*, 91: 435-440.

Hagemann, R. (2004) The sexual inheritance of plant organelles. Molecular biology and biotechnology of plant organelles, H. Daniell and C. Chase, eds (Dortrecht: Springer), pp. 93-113.

Hagemann, R. and Schröder, M. B. (1998) The cytological basis of the plastid inheritance in angiosperms. *Protoplasma* 152: 57-64

Hansen, A. K., Escobar, L. K., Gilbert, L. E.and Jansen, R.K. (2007) Paternal, maternal, and biparental inheritance of the chloroplast genome in Passiflora (Passifloraceae): implications for phylogenetic studies. *American Journal of Botany.* 94: 42-46

Horlow, C., Goujaud, J., Lepingle, A., Missonier, C. and Bourgin, J.P. (1990) Transmission of Paternal Chloroplasts in Tobacco (Nicotiana-Tabacum). *Plant Cell Reports*, 9: 249-252.

Inoue, Y., Suzuki, T., Hattori, M., Yoshimoto, K., Ohsumi, Y. and Moriyasu, Y. (2006) AtATG genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in Arabidopsis root tip cells. *Plant Cell Physiol*, 47: 1641-1652.

Ivanov , S.V.A.V.S.a.K., E. N. (2005) Cumulative effect of low and high atrazine concentrations on Arabidopsis thaliana plants *Russian Journal of Plant Physiology*, 52: 213-219.

Ketelaar, T., Voss, C., Dimmock, S.A., Thumm, M. and Hussey, P.J. (2004) Arabidopsis homologues of the autophagy protein Atg8 are a novel family of microtubule binding proteins. *FEBS Lett*, 567: 302-306.

Klionsky, D.J. (2005) The molecular machinery of autophagy: unanswered questions. *J Cell Sci*, 118: 7-18.

Kuang, A. and Musgrave, M.E. (1996) Dynamics of vegetative cytoplasm during generative cell formation and pollen maturation in Arabidopsis thaliana. *Protoplasma*, 194: 81-90.

Kuroiwa, T. (1991) The Replication, Differentiation, and Inheritance of Plastids with Emphasis on the Concept of Organelle Nuclei. *International Review of Cytology-a Survey of Cell Biology*, 128: 1-&.

Kuroiwa, T., Kawano, S., Nishibayashi, S. and Sato, C. (1982) Epifluorescent microscopic evidence for maternal inheritance of chloroplast DNA. *Nature*, 298: 481-483.

Lee, D. and Natesan, E. (2006) Evaluating genetic containment strategies for transgenic plants. *Trends Biotechnol*, 24: 109-114.

Lee, S.B., Kwon, H.B., Kwon, S.J., Park, S.C., Jeong M.J., Han, S.E., Byun M.Ok and Daniell, H. (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Mol. breed*, 11: 1- 13

Marano, M.R. and Carrillo, N. (1991) Chromoplast formation during tomato fruit ripening. No evidence for plastid DNA methylation. *Plant Mol Biol*, 16: 11-19.

Mascarenhas, J.P. (1989) The Male Gametophyte of Flowering Plants. *Plant Cell*, 1: 657-664.

Medgyesy, P., Fejes, E. and Maliga, P. (1985) Interspecific chloroplast recombination in a Nicotiana somatic hybrid. *Proc Natl Acad Sci U S A*, 82: 6960-6964.

Medgyesy, P., Pay, A., Marton, L. (1986) Transmission of paternal chloroplasts in *Nicotiana*. Mol Gen Genet 204:195-198

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998) Arabidopsis thaliana: A model plant for genome analysis. *Science*, 282: 662-+.

Mitou, G., Budak, H. and Gozuacik, D. (2009) Techniques to study autophagy in plants. *Int J Plant Genomics*, 2009: 451357.

Miyamura, S., Nagata, T. and Kuroiwa,T. (1986) Quantitative fluorescence microscopy on dynamic changes of plastid nucleoids during wheat development. Protoplasma 133: 66- 72

Mogensen, H.L. (1996) The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany*, 83: 383-404.

Nagata, N., Saito, C., Sakai, A., Kuroiwa, H. and Kuroiwa, T. (1999) The selective increase or decrease of organellar DNA in generative cells just after pollen mitosis one controls cytoplasmic inheritance. *Planta*, 209: 53-65.

Natcheva, R. and Cronberg, N. (2007) Maternal transmission of cytoplasmic DNA in interspecific hybrids of peat mosses, Sphagnum (Bryophyta). *Journal of Evolutionary Biology*, 20: 1613-1616.

Neale, D.B., Marshall, K.A. and Sederoff, R.R. (1989) Chloroplast and mitochondrial DNA are paternally inherited in Sequoia sempervirens D. Don Endl. *Proc Natl Acad Sci U S A*, 86: 9347-9349.

Ngernprasirtsiri, J., Kobayashi, H. and Akazawa, T. (1988a) DNA methylation as a mechanism of transcriptional regulation in nonphotosynthetic plastids in plant cells. *Proc Natl Acad Sci U S A*, 85: 4750-4754.

Ngernprasirtsiri, J., Kobayashi, H. and Akazawa, T. (1988b) DNA Methylation Occurred around Lowly Expressed Genes of Plastid DNA during Tomato Fruit Development. *Plant Physiol*, 88: 16-20.

Nishimura, Y., Misumi, O., Matsunaga, S., Higashiyama, T., Yokota, A. and Kuroiwa, T. (1999) The active digestion of uniparental chloroplast DNA in a single zygote of Chlamydomonas reinhardtii is revealed by using the optical tweezer. *Proc Natl Acad Sci U S A*, 96: 12577-12582.

Nishimura, Y., Misumi, O., Kato, K., Inada, N., Higashiyama, T., Momoyama, Y. and Kuroiwa, T. (2002) An mt(+) gamete-specific nuclease that targets mt(-) chloroplasts during sexual reproduction in C. reinhardtii. Genes Dev, 16: 1116-1128.

Nishiyama, R., Ito, M., Yamaguchi, Y., Koizumi, N. and Sano, H. (2002) A chloroplast-resident DNA methyltransferase is responsible for hypermethylation of chloroplast genes in Chlamydomonas maternal gametes. *Proc Natl Acad Sci U S A*, 99: 5925-5930.

Nishiyama, R., Wada, Y., Mibu, M., Yamaguchi, Y., Shimogawara, K. and Sano, H. (2004) Role of a nonselective de novo DNA methyltransferase in maternal inheritance of chloroplast genes in the green alga, Chlamydomonas reinhardtii. *Genetics*, 168: 809-816.

Okumoto, S., Koch, W., Tegeder, M., Fischer, W.N., Biehl, A., Leister, D., Stierhof, Y.D. and Frommer, W.B. (2004) Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. *J Exp Bot*, 55: 2155-2168.

Pacak, A. and Szweykowska-Kulinska, Z. (2003) Organellar inheritance in liverworts: An example of Pellia borealis. *Journal of Molecular Evolution*, 56: 11-17.

Pacini E., T.P.E., Singh M. B., Knox R. B. (1992) Development of Plastids in Pollen and Tapetum of Rye-grass, Lolium perenne L *Ann Bot*, 70: 179-188.

Pandey, K.K. and Blaydes, G.W. (1957) Cytoplasmic Inheritance of Plastids in Impatiens Sultanii Hook, F., Petunia Violacea Lindl. and Cholrophytum Elatum R. Br. Ohio J Sci 57: 135-147

Řepková J. (2010) Potential of chloroplast genome in plant breeding. Genet. Plant Breed. 46: 103-113

Robertson, E.J., Pyke, K.A. and Leech, R.M. (1995) arc6, an extreme chloroplast division mutant of Arabidopsis also alters proplastid proliferation and morphology in shoot and root apices. *J Cell Sci*, 108 (Pt 9): 2937-2944.

Ruf, S., Karcher, D. and Bock, R. (2007) Determining the transgene containment level provided by chloroplast transformation. *Proc Natl Acad Sci U S A*, 104: 6998-7002.

Sakamoto, W., Miyagishima, Sy. and Jarvis, P (2008) Chloroplast biogenesis: control of plastid development, protein import, division and inheritance. The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD.doi:10.1199/tab.0110.

Sikdar, S. R., Serino, G. , Chaudhuri, S. and Pal Maliga (1998) Plastid transformation in Arabidopsis thaliana. Plant Cell rep. 18: 20-24

Svab, Z. and Maliga, P. (2007) Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proc Natl Acad Sci U S A*, 104: 7003-7008.

Szmidt, A.E., Alden, T. and Hallgren, J.E. (1987) Paternal Inheritance of Chloroplast DNA in Larix. *Plant Molecular Biology*, 9: 59-64.

Tang, L.Y., Nagata, N., Matsushima, R., Chen, Y., Yoshioka, Y. and Sakamoto, W. (2009) Visualization of plastids in pollen grains: involvement of FtsZ1 in pollen plastid division. *Plant Cell Physiol*, 50: 904-908.

Testolin, R. and Cipriani, G. (1997) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in the genus Actinidia. *Theoretical and Applied Genetics*, 94,:897-903.

Tewari, K.K. and Wildman, S.G. (1966) Chloroplast DNA from Tobacco Leaves. *Science*, 153: 1269-&.

Toyooka, K., Moriyasu, Y., Goto, Y., Takeuchi, M., Fukuda, H. and Matsuoka, K. (2006) Protein aggregates are transported to vacuoles by a macroautophagic mechanism in nutrient-starved plant cells. *Autophagy*, 2: 96-106.

Van Aelst, A.C., Pierson, E.S., Van Went, J.L. and Cresti, M. (1993) Ultrastructural changes of Arabidopsis thaliana pollen during final maturation and rehydration. *Zygote*, 1:173-179.

Wang, T., Li, Y., Shi, Y., Reboud, X., Darmency, H. and Gressel, J. (2004) Low frequency transmission of a plastid-encoded trait in Setaria italica. *Theor Appl Genet*, 108: 315-320.

Wilson, Z.A., Morroll, S.M., Dawson, J., Swarup, R. and Tighe, P.J. (2001) The Arabidopsis MALE STERILITY1 (MS1) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors. *Plant J*, 28: 27-39.

Xie, Z. and Klionsky, D.J. (2007) Autophagosome formation: core machinery and adaptations. *Nat Cell Biol*, 9: 1102-1109.

Yamamoto, Y., Nishimura, M., Hara-Nishimura, I. and Noguchi, T. (2003) Behavior of vacuoles during microspore and pollen development in Arabidopsis thaliana. *Plant Cell Physiol*, 44,:1192-1201.

Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T. and Ohsumi, Y. (2004) Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. *Plant Cell*, 16: 2967-2983.

Zhang, Q. and Sodmergen (2010) Why does biparental plastid inheritance revive in angiosperms? *J Plant Res*, 123: 201-206.

Acknowledgements

I would like to thank the ministry of high education in Egypt, for funding this scholarship in Germany.

I would like to thank Prof. Dr. Dario Leister for opening his lab to implement this work in.

I am grateful to Dr. Anja Schneider for supervision and discussions.

I would like to thank Prof. Dr. Peter Geigenberger for taking over the second position in the exam commission.

I would like to thank Dr. Cordelia Bolle for proofreading the German summary.

I would like to thank all my colleagues for good contact in science and social events.

Special thanks to Dr. Fatima Chigri, Henning, Michael and Tobias for helping till the last minute.

I would like to dedicate this work to whom lost their lives at 25 Jan 2011 in Egypt to give dignity to all Egyptians all over the world.

To my mother who always gave me the real safety.

To my siblings Safia, Afaf, Atef and Gehan and their families.

To my lovely kids Medo and Rony.

To Shimaa, my better half who shares with me the whole life.

Curriculum vitae

PERSONAL DATA

Name: Ashraf Abdelmontaleb Mohamed Elsayed

Birth date: 01.06.1975

Birth place: Dakahlia

Nationality: Egyptian

RESEARCH EXPERIENCE

Jan 2007- Mach 2011

Ludwig-Maximilians-Universität (München, Germany)

Department Biology I, Botany

PhD thesis in „ Analysis of paternal plastid inheritance in Arabidopsis thaliana and its impact on biosafety of gene-modified plants“

Supervisor: Dr. Anja Schneider

2004

Mansoura University (Mansoura, Egypt)

Faculty of Science

Botany Department

M.Sc thesis in “Potential mutagenecity of some food additives”

EDUCATION

1997

B.Sc (Bachelor in Special Botany)

Botany Department

Faculty of Science

Mansoura University (Mansoura, Egypt)

Ehrenwörtliche Versicherung

Hiermit versichere ich, dass die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Der Autor hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterzeichen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Vorgelegt.

München, März 2011

Ashraf Elsayed