The Egg Apparatus-Specific Gene *ZmEA1* Encodes a Signalling Protein Required for Micropylar Pollen Tube Guidance in Maize (*Zea mays* L.)

Dissertation

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Professor Dr. Arno Frühwald
Dekan

Herewith, I, Dr. Jean Broadhvest, confirm that I have corrected the English of the PhD thesis entitled "The egg apparatus-specific gene *ZmEA1* encodes a signalling protein required for micropylar pollen tube guidance in maize (*Zea mays* L.)" of Mrs. Mihaela Luiza Márton.

Regards,

Jean Broadhvest

to Mihai

and my parents

Contents

Abbreviations

Chemicals and Solutions

CSPD	3-(4-methoxyspiro{1,2-dioxetan-3,2'-(5'chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl) Phenylphosphate, Disodium salt
DIG/Dig	Digoxigenin
DMSO	Dimethylsulfoxyde
DTT	Dithiothreitol
EtBr	Ethidium bromide
SSC	sodiumchloride-sodiumcitrate
TAE	Tris-Acetate-EDTA electrophoresis buffer

Genetics and Molecular biology

aa	amino acids
cDNA	complementary DNA
dag	days after germination
dap	days after pollination
dATP	2'-deoxyadenosine 5´-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2´-deoxyribonucleoside 5´-triphosphates
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
E. coli	*Escherichia coli*
EST	expressed sequence tag
Fig.	figure
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GFP	Green fluorescent protein
GUS	β-Glucuronidase
mRNA	messenger RNA
NOS	nopaline synthase
OCS	octopine synthase
ORF	open reading frame
PAT	Phosphinothricin acetyl transferase
PCR	polymerase chain reaction
pfu	plaque forming unit(s)
Poly(A+) mRNA	polyadenylated mRNA
Q RT-PCR	quantitative reverse transcription polymerase chain reaction
RACE	rapid amplification of cDNA ends

RFLP	restriction fragments length polymorphisms
RNA	ribonucleic acid
RNAse	ribonuclease
RT	reverse transcription
Ubi	ubiquitin
UTR	untraslated region
wt	wild type

Standard Units and Physical Parameters

bp	basepair	ng	nanogram
℃	degree Celsius	nm	nanometer
cm	centimeter	nM	nanomolar
Ci	Curie, unit of radioactivity	Pa	Pascal
g	gram	p.A.	per analyse
h	hour	pg	picogram
kbp	kilobasepair	psi	pound per square inch
kDa	kilodalton	µg	microgram
m	meter	µl	microliter
M	molar	µm	micrometer
min	minute	µM	micromolar
mJ	millijoule	sec	second
ml	millilitre	temp.	temperature
mm	millimeter	Tm	melting temperature
mM	millimolar	U	unit (restriction enzyme)
mmol	millimole	UV	ultraviolet light
mOsm	milliosmol		

Amino Acids

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

1 Introduction

1.1 Female gametophyte development and function

The plant life cycle alternates between a multicellular diploid organism, the sporophyte, and a multicellular haploid organism, the gametophyte. Gametophytes and sporophytes differ morphologically and functionally. After meiosis, the sporophyte gives rise to sexually differentiated types of spores, microspores and megaspores. These spores divide mitotically and develop into gametophytes, whose main function is to produce the gametes. The fusion of male (sperm cell) and female (egg + centrall cell) gametes establishes the next sporophytic generation, thereby completing the life cycle (Raven *et al.*, 1999; Drews *et al.*, 1998).

The gametophytes play a central role in double fertilisation, which is one of the defining features of reproductive development in flowering plants (angiosperms) (Raghavan, 2003). During double fertilisation one sperm cell fuses with the egg cell to give rise to the embryo and the second sperm cell fertilises the central cell to induce endosperm formation (Russell, 1992). The double fertilisation process was first discovered by Nawaschin and Guignard in the liliaceous plants, *Lilium martagon* and *Fritillaria tenella* as well as *L. pyrenaicum* (Nawashin, 1898; Guignard, 1899). In 1901, Guignard was the first who published about this process in maize. Double fertilisation involves a complex series of interactions between essentially three structures, the male gametophyte (MG), the female gametophyte (FG) and the sporophytic tissues of the pistil. These interactions are culminating in the fusion of gametic nuclei and the formation of an embryo and endosperm through separate fusion and activation steps (Weterings and Russell, 2004).

The mature MG, also referred to as the pollen grain or microgametophyte, develops within the anther locules and is composed of two or three haploid cells: one vegetative cell that encloses a generative cell (bicellular pollen grain) or two sperm cells (tricellular pollen grain). In the case of the tricellular pollen grain, sperm cells are formed after the second mitotic division within the anther (as in grasses and crucifers), while in the bicellular pollen grain the second mitotic division occurs during pollen tube (PT) growth as in many Solanaceaes species (McCormick, 1993; McCormick, 2004). The vegetative cell coordinates PT growth and thus the delivery of the two male gametes to the FG. In contrast to most animal and many lower plant species, sperm cells of flowering plants

are non-motile and are transported from the stigma to the FG via the PT to allow double fertilisation (Weterings and Russell, 2004).

The FG, also referred to as the embryo sac or megagametophyte, develops within the ovule, which itself is localised within the carpel's ovary. The FG constitutes the structural setting for double fertilisation. More than 15 different patterns of FG development have been described (Maheshwari, 1950; Koltunow, 1993). The developmental pattern exhibited by most species, including the Poaceae maize (*Zea mays* L.) (Weatherwax, 1919; Cooper, 1937; Kiesselbach, 1999) and *Arabidopsis* (Misra, 1962), is usually referred to as the Polygonum type because it was first described in *Polygonum divaricatum* (Strasburger, 1879). The FG of the Polygonum-type is found in about 70% of the species examined (Maheshwari, 1950) and is thought to be the ancestral type (Huang and Russell, 1992). The development of the Polygonum-type FG occurs over two phases referred to as megasporogenesis (megaspore formation) and megagametogenesis (embryo sac development) (Webb and Gunning, 1990; Huang and Russell, 1992; Yadegari and Drews, 2004). During megasporogenesis, a diploid megaspore mother cell undergoes meiosis and gives rise to four haploid megaspores. The chalazal-most megaspore survives to become the functional megaspore, while the other three microspores undergo programmed cell death. During megagametogenesis, the functional megaspore undergoes three cycles of mitosis, producing an eight-nucleate coenocytium. Phragmoplasts and cell plates form between sister and nonsister nuclei after the third mitosis, and soon, the FG cells become completely surrounded by cell walls (Webb and Gunning, 1994). During cellularization, two nuclei, one from each pole (the polar nuclei), migrate toward the centre of the developing FG. In *Arabidopsis* and other species, the polar nuclei fuse before fertilisation, forming the secondary nucleus (Webb and Gunning, 1994; Christensen *et al.*, 1997). By contrast, in maize and other species, the polar nuclei only partially fuse before fertilisation (Diboll, 1968; Kranz *et al.*, 1998; Fig. 1.1 A). Thus, the monosporic Polygonum-type FG typically consists of seven cells at maturity: three antipodal cells, two synergid cells, one egg cell, and one central cell (Maheshwari, 1950; Drews *et al.*, 1998; Yadegari and Drews, 2004). However, this structure may be modified by cell death or cell proliferation events in various species. For example, in *Arabidopsis*, the antipodal cells undergo cell death before fertilisation (Murgia *et al.*, 1993; Christensen *et al.*, 1997), whereas in grasses, such as maize, the antipodal cells proliferate until a group of about 20 to 60 antipodal cells is formed in the mature embryo

sac (Fig. 1.1 A) (Kiesselbach, 1999; Diboll and Larson, 1966; Huang and Sheridan, 1994).

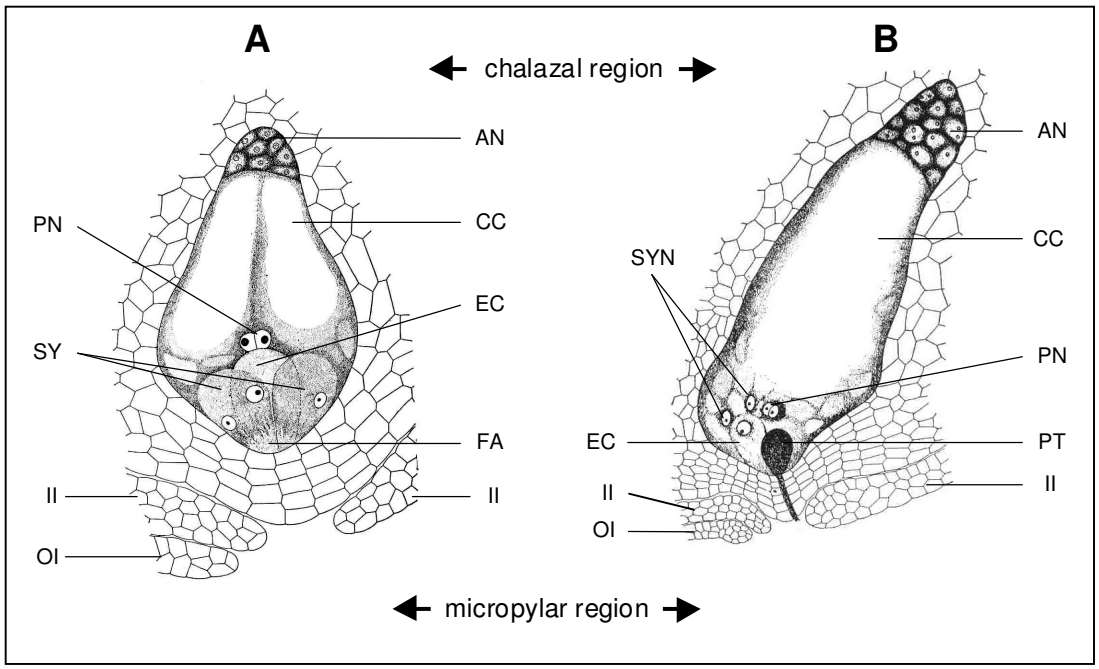

Fig. 1.1: Schematic representation of a mature female gametophyte from maize before and during fertilisation (modified after Kiesselbach, 1999).
(A) Female gametophyte (FG) before fertilisation: egg and synergids represents the egg apparatus, the polar nuclei of the large central cell are partly fused and closed to the egg cell, while antipodals form a group of cells, often containing large nuclei due to endoreduplication. **(B)** A PT entered into FG and its contents are discharged. The sperm cells are still within the cytoplasm of the pollen tube. AN: antipodals, CC: central cell, EC: egg cell, FA: filiform apparatus, II: inner integument, OI: outer integument, PN: polar nuclei, PT: pollen tube, SY: synergids, SYN: synergids nuclei.

Cell-cell interactions play an elementary role in a variety of developmental processes in higher plants either between neighbouring cells or between cells that are separated at some distance (Hülskamp *et al.*, 1995). The exchange of signals between female and male gametophytes must mediate successful fertilisation and is one of the central themes in plant reproductive biology (Shimizu and Okada, 2000). In animal systems, cell-cell communications are extensively studied since some decades and are mainly mediated by signals such as steroids and especially peptide hormones, of which many induce signal cascades after activating receptor tyrosine kinases (RTKs) in the receiving cells. In contrast, cell-cell communication in various biological processes in plants have started to be elucidated in the last few years. The bioinformatical analysis of the first

plant genome (*Arabidopsis thaliana*) resulted in the identification of hundreds of putative membrane bound receptor-like kinases (RLKs) as well as a huge number of highly polymorphic possible peptide ligands (Dresselhaus and Sprunck, 2003). However, fertilisation in flowering plants is a very subtle and accurate process. Compared with animals, in which hundreds of millions of male gametes may target one female gamete (oocyte, ovum or egg cell), in plants, the two nonmotile male gametes target two female gametes with micrometer-level precision (Weterings and Russell, 2004). The FG is enclosed within the nucellus and integuments tissues of the ovule, which themselves are enclosed by the ovary tissue of the pistil and therefore inaccessible for the non-motile MGs. Thus, angiosperms acquired a specialised form of sperm cell delivery: the pollen grain hydrates on the papillar cells of the stigma, where it germinates, and forms a pollen tube (PT) in which the two male gametes are transported over a long distance to the FG. The PT first grows through the stylar transmission tract, probably following attractants produced by the ovule and FG, finally arriving at the FG micropyle. After penetration into the micropylar region of the ovule, the PT delivers its two sperm cells into one of the two synergids by entering near and sometimes penetrating the filiform apparatus of the receptive synergid (Fig. 1.1 B). After the PT ruptures, one of the sperm cells migrates to the egg cell and the second one to the central cell, where their plasma membranes then fuse. Finally, the egg cell nucleus and central cell polar nuclei fuse with one of the two sperm nuclei each and the double fertilisation process is finished. The fertilised egg cell or zygote develops into the diploid embryo, while the fertilised central cell generates a triploid endosperm (Russell, 1992; Olsen, 2004). However, despite a large body of knowledge available concerning morphological description of events regarding the fertilisation process, molecular mechanisms that control these events in plants are still poorly understood (Weterings and Russell, 2004). Many unanswered questions are related to the molecules used for communication between female and male gametophytes as well as with the surrounding maternal tissue.

1.2 Pollen tube guidance

The fertilisation process of plants is governed by cell-cell interactions at different levels. In flowering plants, cell-cell communication is required both for the recognition of the pollen grain by the female reproductive system and to direct the growth of the PT inside the ovary towards the FG. In animals, the nerve growth cone behaviour represents a

particular example of cell-cell interactions that involves the guided growth of cells toward a target (Bixby and Harris, 1991). Pollen tube guidance is a comparable process in higher plants (Huck *et al.*, 2003). Compared to, for example, the well-characterised targeted-directed cell growth in neural systems, the molecular mechanism that guides the growth of PT towards the FG is still on the way to be defined. Cellular guidance involves at least two distinct mechanisms. The guided cell (e.g. pollen tubes in plants) may respond to a gradient of positive or negative signal released by the target (e.g. FG/ovule) or its surrounding cells (Hülskamp *et al.*, 1995). Alternatively, the guided cell may show a polarised growth along a pre-established track (in the case of PTs, for example, a specialised extracellular matrix (ECM) of the pistil; Lord, 2000).

The mechanism that precisely directs the pollen tube through the pistil to the female gametophyte has been studied for more than a century. Chemical attractants from the ovule have been suggested to guide the PT to the FG (Mascarenhas, 1993). Recently, it was demonstrated that PTs of *Arabidopsis thaliana* grow towards the ovule inner integument along a γ-aminobutyric acid (GABA) gradient established by the sporophytic tissues of the stigma (Palanivelu *et al.*, 2003). The *POP2* gene was found to encode a transaminase that degrades GABA and thus contributes to the formation of a GABA gradient from the stigma towards the integuments. However, the pollen tube-attracting activity of GABA could not be reproduced *in vitro*, suggesting that additional molecules are required together with GABA. Other sporophytic molecules involved in guidance through the transmitting tract have been recently identified including arabinogalactans (Sanchez *et al.*, 2004) and small secreted proteins like chemocyanin, a small basic protein from the lily stigma (Kim *et al.*, 2003). However, models have indicated that this distance is far too long to be governed by a single gradient and may require several consecutive cues (Lush, 1999; Weterings and Russell, 2004). To understand the various mechanisms and components of PT guidance, several approaches were performed in different species, especially in the model plant species *Arabidopsis thaliana*, and in *Torenia fournieri*, a plant with a FG protruding from the micropyle of the ovule. Genetic approaches were applied to determine whether and at which steps the PT path may be influenced by the ovules (long-range guidance). PT behaviour was investigated in various sporophytic recessive female-sterile mutants that differ in the extent to which either the sporophytic or the gametophytic tissue of the ovule is affected (Hülskamp *et al.*, 1995). In the *bell* (*bel1*) and *short integuments1* (*sin1*) mutants, with both sporophytic tissue and the embryo sac affected, and in the 47H4 and 54D12

mutants, with sporophytic tissue apparently normal, but with the embryo sac development affected, PTs arrived at the surface of the placenta, even when ovaries did not contain FGs. In all four mutants, it was also noticed that after emerging on the surface of the septum, instead of being directed toward a funiculus and the micropyle of an ovule, PTs grew randomly using all available surfaces, including the ovary wall. These results demonstrate that the directional growth of the PT inside the transmitting tract, as reflected in the pattern of PT emergence, is affected by the presence of intact ovules within the ovary (Hülskamp *et al.*, 1995).

These genetic studies have also shown that ovules lacking FGs fail to attract PTs, supporting the hypothesis that the embryo sac is the source of the attractant that guides the PT to the ovule (Hülskamp *et al.*, 1995; Ray *et al.*, 1997; Shimizu and Okada, 2000). Pollen tubes selectively grew towards ovules that contain a FG in the sporophytic mutant 54D12 (Hülskamp *et al.*, 1995) and in the gametophytic mutant TL-1, in which the sporophytic cells are genetically normal but the FG is absent from about half of the ovules because of a genetic imbalance after meiosis (either all four megaspores die or at least one megaspore may occasionally survive and subsequently divide but the specialised cell types of a normal FG do not appear in these ovules) (Ray *et al.*, 1997). In mutants in which FG development is affected severely (e.g. 54D12 mutants, with ovules arrested during megasporogenesis or early embryo sac development), pollen tubes fail to grow along the funiculus (Hülskamp *et al.*, 1995). These results suggested that guidance by the FG is necessary in *Arabidopsis* from the stage at which the PT grows towards the funiculus of each ovule (Higashiyama *et al.*, 2003). By contrast, in mutants in which FG development is less severely affected (e.g. *magatama* mutants, which exhibit delayed FG development and which display unfused polar nuclei at the time of pollination), PTs grow along the funiculus but do not enter the micropyle (Shimizu and Okada, 2000). All these observations suggest that pollen tube guidance by the female gametophyte in *Arabidopsis* involves at least two phases: guidance from the placenta to the funiculus (funicular guidance phase) and guidance from the funiculus to the micropyle (micropylar guidance phase) (Fig. 1.2; Shimizu and Okada, 2000). However, female gametophyte mutants defective specifically and exclusively in pollen tube guidance have not been up to date reported.

It is still not known whether the signal of funicular guidance is diffusible and directly derived from the FG or an indirect signal that causes a change in the extracellular matrix (ECM) of sporophytic cells (Higashiyama *et al.*, 2003). Funicular guidance

appears to be impaired in *Arabidopsis inner-no-outer* (*ino*) mutants, in which the outer integument does not develop, but the FG develops normally. Both the direct and indirect hypotheses could account for the defect in *ino* funicular guidance. Micropylar guidance does not appear to be defective in the *ino* mutant because it develops seeds, albeit in dramatically reduced numbers (Baker *et al*., 1997). The *pollen-pistil interaction2* (*pop2*)/*pop3 Arabidopsis* mutant is defective in both funicular and micropylar guidance (Wilhelmi and Preuss, 1996). This is a sporophytic mutant that is defective in both male and female tissues. The PT cannot enter the micropyle of the *pop2/pop3* mutant, unlike that of the *ino* mutant, even when growing nearby, probably because of a defect in the ability of the PT to perceive and react to the directional signal (Palanivelu *et al*., 2001).

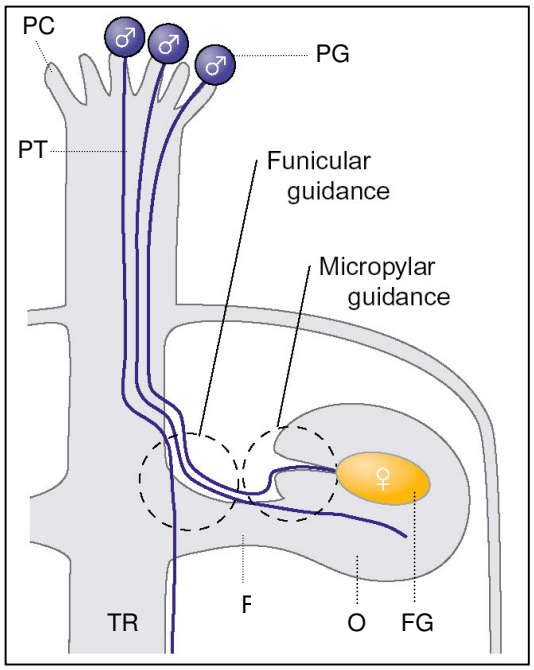

Fig. 1.2: The gametophytic phase of pollen tube guidance in *Arabidopsis thaliana* (modified after Higashiyama *et al*., 2003). Blue solid lines are pollen tubes (PTs) growing from pollen grains (PG, ♂) toward the female gametophyte (FG, ♀). Some PTs lose their way in the regions surrounded by dashed circles when the FG is defective, suggesting the existence of directional signals that emanate from the FG in these regions. In *Arabidopsis*, PT guidance by the FG is governed by at least two processes: funicular guidance and micropylar guidance. F: funiculus, O: ovule, PC: papillar cell, TR: transmitting tract.

With the aim to dissect the origin and nature of the FG guidance signal, Higashiyama and colleagues (1998) performed laser ablation experiments using an *in vitro* pollen germination system and ovules from *Torenia fournieri*. The results indicated that the synergids are the source of a PT attraction signal and the competence of PTs to

respond to this directional signal requires growth within gynoecial tissues. Laser cell ablation of individual cells of the naked embryo sac demonstrated the existence of a short-range diffusible attraction signal, some 100-200 µm around the egg apparatus (Higashiyama *et al.*, 2001). These signalling molecules seem to be species specific in *Torenia fournieri* and closely related species (Higashiyama *et al.*, 2003). Additionally, PTs grew normally through the transmitting tissue but rarely arrived at the funiculus and did not enter the micropyle of FGs in interspecific crosses using *Arabidopsis* and other species of the Brassicaceae (Shimizu and Okada, 2000). These data suggest a role for pollen guidance in the species barrier concept involving short-range guidance signals.

Genetic and physiological studies thus predict the existence of mechanisms for a long- and a short-range PT guidance that may involve signalling molecules generated by both sporophytic and gametophytic tissues. Pollen tubes are neither attracted to immature or incompletely formed ovules nor to ovules lacking embryo sacs. The mature, fully formed FG was shown to be necessary to guide the PT from the placental tissue to the ovule, a distance of attraction not exceeding ~200 µm in *Arabidopsis*. It was suggested that the FG egg apparatus, composed of synergid and egg cells, is responsible for funiculus guidance, and possibly also for micropyle guidance. The female gametophyte thus produces at least two directional signals, and at least one of them is diffusible and derived from the two synergid cells and/or the egg cell (Hülskamp *et al.*, 1995; Ray *et al.*, 1997; Higashiyama *et al.*, 2003; Weterings and Russell, 2004; Shimizu and Okada, 2000). But the biochemical nature of the guidance cue is still unknown. Calcium (Ca^{2+}) was first proposed to be a guidance signal because it can attract PTs *in vitro* in some species (Reger *et al.*, 1992). Ca^{2+} is also present at high concentrations in synergid cells (Tian and Russell, 1997) and was shown to be required for PT growth (Li *et al.*, 1999). However, the addition of Ca^{2+} to the medium of the *in vitro T. fournieri* system did not affect PT attraction and PTs of different *Torenia* species failed to find ovules of other *Torenia* species, indicating that calcium is neither a universal nor a specific attractant (Higashiyama, 2002; Higashiyama *et al.*, 2003; Yadegari and Drews, 2004). The role of Ca^{2+} as a second messenger in signal transduction mechanisms may provide amplification of more specific attraction systems (Weterings and Russell, 2004). A molecule such as a peptide that is synthesised by the cells of the egg apparatus is a more likely candidate as the attractant. Peptides readily evolve and therefore are good candidates providing a species-specific signal (Higashiyama *et al.*, 2003). Current knowledge about the functional roles of plant peptides is very little, but they could

function in a similar manner as animal peptides, where they regulate a huge variety of biological processes. Especially signalling peptides are key molecules of cell-cell communication as they have the potential to induce whole signal transduction cascades of many biological processes. Due to their small size (ORFs smaller than 100 aa), peptide genes mostly have been overlooked during genome sequence annotation, as a size of >100 aa was used as a criteria to postulate an ORF of a novel gene (Zhu *et al.*, 2003). Additionally, the expression of many peptide genes is probably often limited to few specialised plant cells (e.g. *ZmES1-4*, Cordts *et al.*, 2001; *CLAVATA3*, Fletcher *et al.*, 1999) and this might have entangled their identification by molecular and biochemical methods (Dresselhaus and Sprunck, 2003).

Synergid degeneration is considered to be essential for fertilisation and in many species the receptive synergid degenerates before the PT reaches the FG (Russell, 1992). During the last two years, several FG mutants affecting the fertilisation process have been reported, including the *Arabidopsis* mutants *gametophytic factor2* (*gfa2*), *feronia* (*fer*), and *sirene* (*srn*) (Christensen *et al.*, 2002; Huck *et al.*, 2003; Rotman *et al.*, 2003). Pollen tubes were attracted to the FG of these mutants, but fertilisation did not take place. In the *srn* and *gfa2* mutants, displaying normal or essentially normal embryo sac development (*gfa2* FGs have defects in fusion of the polar nuclei), synergids did not degenerate after pollination but are still capable to attract pollen tubes (Rotman *et al.*, 2003; Christensen *et al.*, 2002). These observations suggest that, at least in *Arabidopsis*, synergid cell death seems not to be required for PT attraction. The *GFA2* gene was isolated and shown to be expressed throughout the plant. *GFA2* encodes a DnaJ-like chaperone that functions in the mitochondrial matrix, and is the *Arabidopsis* ortholog of yeast Mdj1p, which is required for mitochondrial function. These data suggest that synergid cell death requires functional mitochondria, which also are required for cell death in animals (Christensen *et al.*, 2002). Interestingly, when a wild type PT entered the receptive synergid of a *fer* and a *srn* mutant female gametophyte, it continued to grow, failed to rupture and to release the sperm cells, and therefore invaded the embryo sacs. PT reception is achieved within the synergid by an immediate interaction of the PT with the FG. Because there is no obvious developmental defect in mutant *feronia* FGs and synergids undergo normal differentiation and degeneration, the defect in PT reception observed in the *fer* mutant must, therefore, be the result of a deficient synergid function, i.e. a failure in the communication between the synergid and the PT (Huck *et al.*, 2003). Up to date, isolation and characterisation of the *FER* and

SRN genes was not accomplished. Nevertheless, these data suggest already that the arrest of PT growth and rupture are controlled directly or indirectly by a compound from the synergid. Huck *et al.* (2003) have also shown that two or more PTs can enter mutant FGs in *Arabidopsis feronia* mutants. Therefore, it is likely that the expression of a synergid-borne attractant persists in *fer* mutants and results in the attraction of supernumerary PTs. The fertilised FG of *Torenia fournieri* no longer attracts PTs, although one synergid always remains after PT discharge (Higashiyama *et al.*, 2001). This cessation of PT attraction might be involved in blocking polyspermy. It seems that a series of blocks controls the distribution of PTs to consecutive ovules of a pistil and prevents polyspermy during the fertilisation process of higher plants. However, it is still not known whether secretion of the attractant from the synergid cell simply stops after fertilisation or whether a repulsive signal or a decomposing molecule affects PT attraction by fertilised female gametophytes (Higashiyama *et al.*, 2003).

Biochemical approaches have been unsuccessful to date to identify the signalling molecules secreted by the FG, as the FG consists only of a few cells and typically is embedded in several cell layers of maternal tissues, thus recalcitrant for experimental analyses. Up to now, the molecular nature of gametophytic attractants was also not described, but analysis of genes that disrupt FG function without otherwise affecting their development might help in dissecting the mechanisms that regulate gamete delivery (Weterings and Russell, 2004). Relatively few genes expressed in the FG have been identified and characterised so far. Some genes are involved in the cell cycle (e.g. *AtMCM7*, Springer *et al.*, 2000; *ZmMCM6* and ribosomal proteins, Dresselhaus *et al.*, 1999a; *AtRbr1*, Ebel *et al.*, 2004) or in gene regulation (e.g. MADS box transcription factors, Heuer *et al.*, 2001; *AGL15*, Perry *et al.*, 1996). Other genes play unknown roles in cell differentiation and fertilisation, such as *ZmTLA1* (*Zea mays Transparent Leaf Arrea1*, Dresselhaus *et al.*, unpublished) and *ZmES1-4* (*Zea mays Embryo Sac1-4*, Amien, 2003). Genes controlling endosperm development in *Arabidopsis* such as *FIS2* (*Fertilisation-Independent Seed2*, Luo *et al.*, 2000), *MEA* (*Medea*, Grossniklaus *et al.*, 1998) and *ZmEBE-1/2* (*Zea mays Embryo sac/Basal endosperm transfer layer/Embryo surrounding region-1/2*, Magnard *et al.*, 2003) have been identified as being expressed in the central cell prior fertilisation. However, FG-specific genes that could play an important role in PT guidance have not been identified up to date.

1.3 Aims of the work

The aim of the present work is to identify genes from maize that could play important roles in cell-cell communication during the fertilisation process, especially in pollen tube guidance. ESTs should be generated from an egg cell-specific cDNA library (Dresselhaus *et al.*, 1994) and genes that are highly and specifically expressed in the female gametophyte and encode small proteins (ORF smaller than 100 aa) with N-terminal signal peptides or transmembrane domains should be selected for further functional analysis. The promoter of one candidate gene should be isolated and used to generate GUS promoter deletions constructs for studying promoter specificity and activity in maize and rice ovules. Plants expressing a GFP fusion protein should be generated to localise the candidate protein in maize using the endogenous functional promoter. Additionally, RNAi and antisense constructs should be generated to knock down the candidate gene activity in maize with the goal to study the function of the gene.

2 Materials and Methods

2.1 Chemicals, enzymes and other materials

Unless mentioned, “p.A.” purity grade chemicals and deionised water (Milli-Q Plus Water system, Millipore, Bedford, U.S.A.) were used for preparation of all solutions and media. According to different requirements, solutions and media were autoclaved (20 min, 120°C, 2 x 10^5 Pa) or filter sterilised (0.22 µm Millex® GP or Steritop™, Millipore, Bedford, U.S.A.).

General lab chemicals were purchased from the following companies: AgrEvo (Düsseldorf), Applichem (Darmstadt), Biomol (Hamburg), Brand (Wertheim/Main), Duchefa (Harlem, The Netherlands), Fluka (Buchs), Merck-Schuchard (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma-Aldrich (München).

Restriction endonucleases, dNTPs, DTT, DNA polymerases, DNA/RNA modifying enzymes and other proteins were used from Invitrogen (Karlsruhe), MBI-Fermentas (St. Leon-Rot) and New England BioLabs (NEB, Frankfurt am Main).

Gel-blotting papers were used from Schleicher & Schüll (Dassel) and thermal papers for photographic documentation of agarose gels were from Mitsubishi (Kyoto, Japan). Hybond™ N+ and Hybond™ NX nylon membranes, radionucleotides [α-^{32}P]-dCTP (6,000Ci mmol^{-1}) and Hyperfilm™-MP X-ray films, were purchased from Amersham Pharmacia Biotech (Freiburg).

2.2 Plant material

The following maize (*Zea mays* L.) lines were used for this study: inbred lines A188 (Green and Philips, 1975), H99 (D´Halluin *et al*., 1992), B73 (Iowa State University, Ames, U.S.A.), He89 derived line (Mórocz, 1990) and recombinant inbred families, CO159 x TX303, CM37 x T232 (Burr and Burr, 1991). Maize plants were grown under standard green house conditions, with 16 hours light of 24,000-25,000 Lux intensity at 24°C and 8 hours darkness at 20°C, under 55-95% relative humidity. To prevent uncontrolled pollination, ears were covered with bags before the appearance of silks. Flowering plants were manually pollinated or directly used for molecular and cytological analyses.

Plant material from rice (*Oryza sativa*, japonica cultivar variety, inbred line M202), *Tripsacum* (*Tripsacum dactyloides*), barley (*Hordeum vulgare*, Igri variety), *Arabidopsis* (*Arabidopsis thaliana*, Columbia variety) and tobacco (*Nicotiana tabaccum*, SR1 variety) was sampled from greenhouse grown plants.
Onion peels (*Allium cepa* L., a sort from REWE-HANDELSGRUPPE GmbH (Köln)), were used for transient transformation studies.

2.3 Bacterial strains, phages and vectors

E. coli – strains:

- DH5α (Hanahan, 1983)
- DH10B (Grant *et al.*, 1990)
- Electroporation-Competent Cells (Stratagene, La Jolla, U.S.A.)
- SOLR™ (Stratagene, La Jolla, U.S.A.)
- TOP 10 (Invitrogen, Karlsruhe)
- TOP 10F‘ (Invitrogen, Karlsruhe)
- XL1-Blue-MRF‘ (Bullock *et al.*, 1987)

Phages:

- ExAssist™ Interference Helper Phage (Stratagene, La Jolla, U.S.A.)

Vectors:

- p202/4 (containing *ZmEC 135* clone; Cordts, 2000)
- p35S-PAT (Hoechst AG, Frankfurt)
- pBi121 (Clontech, Palo Alto, U.S.A.)
- pBluescript SK+/- (Stratagene, La Jolla, U.S.A.)
- pCR® 2.1-TOPO® (Invitrogen, Karlsruhe)
- pCR®-Blunt II-TOPO® (Invitrogen, Karlsruhe)
- pDE4 (Bayer BioScience, Gent, Belgium)
- pTLH221 (Bayer BioScience, Gent, Belgium)
- pLitmus™ 29 (NEB, Frankfurt am Main)
- pLNU-AB (DNA Cloning Service, Hamburg)
- pMon30049 (Pang *et al.*, 1996)
- pUbi-AB (DNA Cloning Service, Hamburg)
- pUbi-iF2 (DNA Cloning Service, Hamburg)

2.4 Oligonucleotides

Synthesis of oligonucleotides (primers) used in this work was conducted either by Invitrogen (Karlsruhe) or by Sigma-Aldrich (München) and their sequence is given below, in 5'-3' orientation.

Vector primers and sequencing primers

BT	CAGGAAACAGCTATGACC
MB	GTAAAACGACGGCCAGTG
M13 Forward (-20)	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
NosU	CAAGACCGGCAACAGGATTC
OR	ATGCGATCATAGGCGTCTC
P1	ACTAATCCAAGCTGCCACGAC
P4	TTGCCTGCATTGCGATGCATG
P5	CTATCAGGTTTCGACGTCCGA
SK	CGCTCTAGAACTAGTGGATC
Ubi1	ACTGTTTCTTTTGTCGATGC

Primers for 5'-RACE, "Genome Walking" and intron analyses

Adapter 2R	5'–CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT–3'
	3'–H_2N-GCCGGCTCCA-PO_4–5'
AP1	CTAATACGACTCACTATAGGGC
AP2	AGCGTGGTCGCGGCCGAGGT
CML5	ACGATCACTTGCTCACAGTCACAGCTAG
CML6	ATCGCTCTCCTCCAACTAAGC
CML7	GCTGCTTAGTTGGAGGAGAGCGATCGGCT
Long primer	GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
P6	GACCAGGGTCGGACGTCGAAACCTGATAG
P7	TGACCACGCTAACGAAGAGCCCTAGTCGC
P8	TCCACACGATTCTGCCTGCAT
Short primer	GTAATACGACTCACTATAGGGC

Primers used for cloning

EAF-GFP	CGCGACTAGTTCTGCATGTCATCC
EAR-GFP	CGAGGATCCCGCTAGCGATCGAAC
GFPXho	CTCGAGTCACTTGTAGAGTTCATCC
T6Bam	GGATCCGACAACATCGGCATAC

T6Bsr	TGTACAGCTCTCCTCCAACT
T6Mfe	AATTGCTCTCCTCCAACTAAGC
T6Mlu	ACGCGTCCGACAACATCGG
ZE-Bbs	CTCACTCACGAAGACGACATGCAGAATTCAGCGTC
ZE-Not	CTCACTCCGCGGCCGCGGGCTAGCGATCGAACAGGCAG
ZE-Sac	CTCAGCTCTAGAGCGAGCTCGTCCACACGATTCTGCCTGCAT
ZE1-XSma	CTCACTCCCCCCGGGGGGATCCACACGATTCTGCCTGCAT
ZE2-XSma	CTCACTCCCCCCGGGGGGAGTGTTGTGCGCACTCAGGTC
ZE3-XSma	CTCACTCCCCCCGGGGGGACGGATTGCTGGTAGTGGACG

Primers for RT-PCRs and Southern blot-probes

200a	CCCTTGGATTGGATTGGATCG
200b	ACCACCGGTTTCCTGCTGTC
CML8	CGACGCATAGAGTAACATCTC
Gap1	AGGGTGGTGCCAAGAAGGTTG
Gap2	GTAGCCCCACTCGTTGTCGTA
GAPDH-japonica	AGGGTGGTGCCAAGAAGTTCG
GAPDH-O.s.2	GTAACCCCACTCGTTGTCGTA
GUS rev	GTCTGCCAGTTCAGTTCGTTG
Oligo $(dT)_{18\text{-}25}$	$(T)_{18\text{-}25}$
Qa	CATCGTCAACATGAAGGACGA
Qbr	ACGACGATCACTTGCTCACAG
RT1	AGCGCCCGCTGTCCATTCAT
RT2	ATGCCCAATTAACACAACACGC
ZE-GFP1	GAGGAACTGTTCACTGGCGT
ZE-GFP2	CCAGGGTATCACCTTCGAAC

2.5 Standard molecular biological methods

Standard molecular biological methods, such as gel electrophoresis, quantification of nucleic acids, DNA-cloning, etc. were carried out, unless specifically mentioned, as described by Sambrook *et al*. (1989). Unless mentioned, kits and enzymes were used following the manufacturer's recommendations. Plasmid DNA isolation was routinely made with E.Z.N.A® Plasmid Miniprep Kit I and II (Peqlab, Erlangen). Agarose gel electrophoresis were done with 1xTAE buffer and 250 ng EtBr per ml gel. After electrophoresis, DNA fragments were photographed using an UV transilluminator with

the Gene Genius Bio Imaging System (Syngene, Cambridge, U.K.). Pictures were documented on thermal papers (Mitsubishi, Japan) and processed with GeneSnap software (Syngene, Cambridge, U.K.). Electrophoretic DNA controls used were the Lambda DNA/Eco 471 (AvaII)-Marker13 (MBI Fermentas, St. Leon-Rot) for general agarose gels and the “DIG VII-Marker” (Roche, Mannheim) for detection after Southern-blot-transfer.

2.6 Standard PCR

All PCRs (Rybicki, 1996) were performed in a TRIO-Thermoblock cycler or in a TGradient cycler (both Biometra, Göttingen). A 50 µl standard reaction mix was used which comprised: 10-50 ng plasmid DNA or 100-500 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 nM of each forward (5')- and reverse (3')-specific primers, 200 µM dATP, dCTP, dGTP and dTTP, 1-2 U *Taq*- or *Pfu*-DNA-polymerase. If necessary, reaction mixtures were covered with 1-2 drops of mineral oil. PCR reactions made without usage of antibodies against DNA-polymerases were first put in the thermocycler when this one had reached a temperature of around 80°C. A standard PCR protocol consisted of: one preheating step (2 min, 96°C) followed by 30 cycles of denaturation (45 sec, 96°C), primer annealing (45 sec, Tm of primer pairs) and polymerisation steps (1 min (*Taq*-) or 2 min (*Pfu*-DNA-polymerase) per 1 kb amplikon, 72°C). The PCR reactions were always terminated by a final polymerisation step (3–10 min, 72°C) and cooling to 4°C.

Multiplex PCRs in this study were performed using two pairs of primers per PCR reaction and the same standard conditions as described above.

PCR products were analysed by agarose gel electrophoresis as described in 2.5.

2.7 *In vivo* excision of a maize egg cell cDNA library

In vivo excision of a maize egg cell cDNA library (Dresselhaus *et al*., 1994) was performed according to the protocol provided with the Lambda ZAP® II Predigested Vector Kit (Stratagene, La Jolla, U.S.A.), using the ExAssist™ Helper Phage, the *E. coli* strains XL1-Blue-MRF‘ and SOLR™ (all from Stratagene, La Jolla, U.S.A.). Bacterial clones were randomly picked for generation of an egg cell specific EST database.

2.8 DNA sequencing and sequence analysis

EST sequences were generated by Agowa GmbH (Berlin) using the vector primer SK. Clustering with CAP3 algorithm, classification and functional analysis with the BLASTX algorithm (Altschul *et al.*, 1997) at Swiss-Prot and TrEMBL databases of the ESTs were performed by Laurent Viau and Aurélie Defferrard (Bayer CropScience, Evry, France).
Non-EST sequences were generated by DNA Cloning Service (Hamburg) using the *Taq* DNA polymerase FS Cycle Sequencing Kit (Applied Biosystems, Foster City, U.S.A.) and 373A and 377 automated DNA sequencer (Applied Biosystems, Foster City, U.S.A.). DNA and amino acid sequence data were further processed using the Lasergene program software package (DNASTAR Incorporated, Madison, U.S.A.). DNA and protein database searches and sequences comparisons were performed using FASTA and BLAST algorithms (Pearson, 1990) to scan the following databases: GenBank, UniGene (both http://www.ncbi.nlm.nih.gov/), EMBL (http://www.ebi.ac.uk/embl/), DDBJ (http://www.ddbj.nig.ac.jp/), Swiss-Prot and TrEMBL, and tools including SignalP and PSORT from ExPASy Proteomics server (http://us.expasy.org/). *ZmEA1* promoter elements were identified using the databases PLACE (Higo *et al.*, 1999; http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (Lescot *et al.*, 2002; http://intra.psb.ugent.be:8080/PlantCARE/). Secondary and tertiary structure prediction was performed at http://insulin.bio.warwick.ac.uk, with PSIpred (protein structure prediction server) at http://bioinf.cs.ucl.ac.uk/psipred, PDB (protein data bank) at http://pdb.ccdc.cam.ac.uk and SOSUI at http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html.

2.9 5’-RACE

5’-RACE (rapid amplification of cDNA ends) was performed to isolate a full-length cDNA of *ZmEA1* from a cDNA of maize zygotes, provided by Dr. Sprunck (University of Hamburg) and made from 10 zygotes, 16 to 18 hours after *in vitro* pollination using the SMART™ cDNA synthesis Kit (Clontech, Palo Alto, U.S.A.) according to the user manual. Following the protocol prescriptions from the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, U.S.A.), the missing 5'-end of *ZmEA1* cDNA was amplified from 10 ng/µl of the maize zygotes cDNA using the *ZmEA1*-specific primer CML5 and the UPM (universal primer mix), a mixture of two specific RACE-primers

(Long primer and Short primer) in a standard PCR reaction with the following profile: 2 min at 94°C followed by 5 cycles for 30 sec at 94°C and 3 min at 72°C, 5 cycles for 30 sec at 94°C, 30 sec at 70°C and 3 min at 72°C, 20 cycles for 30 sec at 94°C, 30 sec at 68°C and 3 min at 72°C, with a final extension for 10 min at 72°C. PCR products were cloned using the TOPO TA Cloning® Kit (Invitrogen, Karlsruhe) into the vector pCR®2.1-TOPO® (2.3) according to the manufacturer's specifications and sequenced with primers M13 Reverse and M13 Forward (-20).

2.10 Promoter isolation by "Genome Walking"

A "Genome Walking" technique was used to isolate the promoter of the *ZmEA1* gene. Maize A188 GenomeWalker-libraries DNA were constructed following the protocol prescriptions from the Universal GenomeWalker™ Kit (Clontech, Palo Alto, U.S.A.) by using "blunt-end" restriction enzymes (*Eco*RV, *Dra*I, *Hinc*II, *Pvu*II, *Sca*I and *Sma*I) and ligation of the GenomeWalker Adapter (Adapter 2R). These genomic DNA-libraries served as templates for the isolation of the *ZmEA1* promoter sequence by two rounds of genome walking. Each round included two PCR steps, which were performed according to the manufacturer's instructions, with the exception that 5% DMSO was added to the reaction mixes. In the first round, amplifications for the primary PCR were carried out with the outer adapter primer AP1 and outer *ZmEA1*-specific primer CML5. The nested adapter primer AP2 and the nested *ZmEA1*-specific primer CML7 were used for the secondary or "nested" PCR. PCR products were cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Karlsruhe) into the vector pCR®-Blunt II-TOPO® (2.3) and sequenced with primers M13 Reverse and M13 Forward (-20). The second round of genome walking used the *ZmEA1*-specific primers P7 and P6 to extend the promoter sequence identified in the first round. Using the information from the genome walking, the *ZmEA1* promoter sequence was amplified together with a part of its cDNA from genomic DNA using the primers P8 and Qbr. The PCR fragment obtained was cloned using the TOPO TA Cloning® Kit (Invitrogen, Karlsruhe) into the vector pCR®2.1-TOPO® (2.3) to generate plasmid **pZmEA1-P8-Qb**. This plasmid was sequenced using the primers M13 reverse, M13 Forward (-20), P5, P4 and P7.

2.11 RT-PCR, single cell RT-PCR and Q RT-PCR analyses

Multiplex RT-PCR and multiplex single cell RT-PCR (SC RT-PCR) analyses were performed according to Cordts *et al*. (2001), using SuperScript™ II RNase H⁻ Reverse Transcriptase and RNaseOut™ Recombinant Ribonuclease Inhibitor (both Invitrogen, Karlsruhe). Multiplex RT-PCR was performed on 1 µg of total RNA isolated from different maize tissues (RNAs kindly provided by Dr. Heuer and Dr. Dresselhaus, University of Hamburg) using the *ZmEA1*-specific primers RT1 and Qbr. RNA samples were treated before first strand cDNA synthesis with DNase I Amplification Grade (Invitrogen, Karlsruhe) to avoid DNA contamination, according to the manufacturer's recommendations. The multiplex SC RT-PCR analyses were performed with isolated egg cells from maize inbred line A188 using *ZmEA1*-specific forward primers RT1 and RT2, as well as Qbr, as reverse primer. Concurrent amplification of the maize *GAPDH* gene with primers Gap1 and Gap2 was always used as an internal control in both above analyses, as described by Richert *et al*. (1996). PCRs results from both analyses were controlled by agarose gel-electrophoresis and specificity and quantification of the products from the multiplex RT-PCR analysis were compared by hybridisation with a Qa-Qb DIG-dUTP-labelled *ZmEA1*-specific probe (2.12).

Expression levels of the pCMG1 transgene were compared between three transgenic pCMG1 rice lines (2.16.2) and wild-type (M202) rice inbred line by performing an RT-PCR analysis. cDNAs were generated from Poly(A)$^+$ mRNA extracted from 10 pistils per rice line using the Dynabeads® mRNA DIRECT™ Micro Kit (Dynal, Oslo, Norway). Immediately after isolation, poly(A)$^+$ mRNA was used for first strand cDNA synthesis for 50 min at 42°C, using an oligo $(dT)_{18}$ primer. Reverse transcription was conducted in a 20 µl reaction containing 1X First strand buffer, 0.4 mM dNTP's, 50 U SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase, 20 U RNaseOut™ Recombinant Ribonuclease Inhibitor and 10 mM DTT (all Invitrogen, Karlsruhe). Finally, two standard PCRs were separately performed with each cDNA using pCMG1-specific primers RT1 and GUS rev in one reaction and *GAPDH* rice-specific primers GAPDH-japonica and GAPDH-O.s.2 in the other reaction. Specificity and quantification of the products were compared by hybridisation with the RT1-GUS DIG-dUTP-labelled probe and with GAPDH DIG-dUTP-labelled probe (both 2.12), respectively.

Single cell Q (quantitative) RT-PCR analysis was performed with isolated egg cells from a maize line containing pZE-RNAi construct (2.16.1) and from maize inbred line A188.

Reverse transcription was performed for 75 min at 50°C using RevertAid™ H Minus M-MuLV Reverse Transcriptase and RNase inhibitor (both MBI-Fermentas, St. Leon-Rot), together with *ZmEA1*-specific primer CML8 and *GAPDH*-specific primer Gap2 in the same reaction, according to the protocol described by Richert *et al*. (1996). RT products were split by two and separately Real-Time PCR amplified using an iCycler iQ™ machine and an iQ™ SYBR® Green Supermix (both BIO-RAD, München), according to the manufacturer's recommendations. The *ZmEA1*-specific primers RT1 and CML8 (400 nM each primer) were used in one PCR reaction and maize *GAPDH*-specific primers Gap1 and Gap2 (400 nM each primer) or *ZmES1*-specific primers 200a and 200b (400 nM each primer) in the other reaction. PCRs results were controlled by agarose gel-electrophoresis and processed with the iCycler iQ™ Real-Time Detection System Software, version 3.0 for Windows (BIO-RAD, München). *GAPDH*- and *ZmES1*-specific PCR products were used to normalise *ZmEA1* transcript amounts.

2.12 Radioactive- and DIG-labelling of DNA fragments

Generation of radioactive-labelled probes was made in two steps. In the first step, a DNA fragment was amplified via a standard PCR (2.6), separated in a 1% agarose gel and purified with EasyPure® DNA Purification Kit (Biozym, Oldendorf). In the second step, incorporation of [α-^{32}P]-dCTP (6,000 Ci mmol^{-1}) was done using the Prime-It® II Random Primer Labelling Kit (Stratagene, La Jolla, U.S.A.). Separation of unincorporated nucleotides and oligonucleotides was performed with NucTrap™ Push columns (Stratagene, La Jolla, U.S.A.). Incorporation levels were assayed with Wallac 1409-Liquid Scintillation Counter (Roth, Karlsruhe). Probes with at least 30% incorporated radioactive labelled nucleotides were used for hybridisations.

Digoxigenin-11-dUTP (DIG-dUTP)-labelled DNA probes were made by a standard PCR (2.6) and adding Dig-11-dUTP (Roche, Mannheim) to a final concentration of 200 µM. PCR products were separated and purified as described above. The DNA concentration was determined by comparison of fluorescence intensity of fragments in EtBr-agarose gels as well as by spectrophotometric measurements.

All DNA probes used in this work are shown in Table 2.1.

Table 2.1: DNA probes used in this work.

DNA template, primers with specific annealing temperature and size of resulting labelled probes are given. (temp.: temperature, bp: base pairs)

Name of the hybridisation probe	Template	Primer pair	Annealing temp. (°C)	Size (bp)
Qa-Qb	p202/4 (2.3)	Qa/Qbr	58-64	301
RT1-GUS	pCMG1 (2.15)	RT1/GUS rev	58	532
ZE-GFP1-2	pZE-GFP (2.15)	ZE-GFP1/ZE-GFP2	54	346
GAPDH (provided by Dr. Sprunck)	cDNA of *GAPDH*	Gap1/Gap2	58-64	622

2.13 Southern blot analysis

10-25 µg genomic DNA, isolated after Palotta *et al*. (2000), was restricted with endonucleases and separated in 0.8 % agarose gels. PCR-fragments were separated in 1-2% agarose gels. Gel treatments for Southern blots were performed as described in Sambrook *et al*. (1989). DNA was transferred onto Hybond™ NX nylon membranes by capillary transfer (20x SSC) and fixed to membranes with 120 mJ using Stratalinker™ 1800 UV crosslinker (Stratagene, La Jolla, U.S.A.). Alternatively, DNA was transferred onto Hybond™ N^+ nylon membranes by alkaline capillary transfer (0.4 M NaOH) without any further fixation step.

Hybridisation with radioactive probes was done overnight in QuickHyb buffer (Stratagene, La Jolla, U.S.A.) containing 100 µg/ml salmon sperm DNA, as described in the accompanying protocol. Membranes were washed under stringent (65 °C or 68 °C) or non-stringent conditions (60 °C), as follows: 10 min in 2x SSC/0.1 % SDS, 10 min in 1x SSC/0.1 % SDS, 10 min in 0.5x SSC/0.1 % SDS and 10 min in 0.2x SSC/0.1 % SDS solution, as a final washing step. Hybridisation signals were detected by exposing the Hyperfilm™ MP X-ray films with the membranes from 1 day to up to several days at -70 °C, using intensifier screens.

Hybridisation with DIG-labelled DNA probes (20-25 ng/ml hybridisation solution) was performed at 42°C using DIG Easy Hyb solution (Roche, Mannheim). Chemiluminescence detection was done with CSPD® substrate (Roche, Mannheim)

according to the manufacturer's prescriptions. Detection of hybridisation signals was done directly by exposing Hyperfilm^{TM} MP X-ray films with membranes from 5 min to up to several hours at room temperature or at 37°C.

2.14 Genomic mapping

Mapping of *ZmEA1* gene in the maize genome was performed after Burr and Burr (1991). In order to identify RFLPs (Restriction Fragments Length Polymorphisms) between the members of the maize inbred mapping populations CO159 x TX303 and CM37 x T232 (Burr and Burr, 1991), Southern blot analyses (2.13) were performed with 10 µg DNA per sample after restriction of DNA with the endonucleases *Bam*HI, *Bgl*II, *Hind*III, *Kpn*I and *Pst*I. Hybridisation was performed with a Qa-Qb radioactive-labelled *ZmEA1*-specific probe (2.12). The resulting polymorphism's were scored and placed on the Brookhaven National Laboratory map using the Map-Maker program (Burr and Burr, 1991).

2.15 Generation of constructs

pCMG1, **pCMG2** and **pCMG3 (*ZmEA1*p::*GUS*::*NOS*t):** these constructs contain the *GUS* coding sequence under the control of the *ZmEA1* promoter deletion fragments, ZE1 (1570 bp; includes the full length promoter), ZE2 (1013 bp) and ZE3 (470 bp), respectively, and the NOS terminator from *A. tumefaciens*. *ZmEA1* promoter fragments were amplified from the plasmid pZmEA1-P8-Qb (2.10) by standard PCRs using ZE-Bbs as a 3'-primer and ZE1-Xsma, ZE2-XSma or ZE3-XSma as 5'-primers, respectively. PCR products were blunted with the T4 DNA polymerase and cloned into blunted *Xho*I-*Sac*I sites of the vector pBluescript SK+. After cloning, promoter deletions fragments were restricted from the intermediate vectors with *Bbs*I and *Xma*I and cloned into the *Nco*I and *Xma*I sites of the vector pDE4 (*35S*p::*GUS*::*NOS*t, 2.3), generating pZEG1, pZEG2 and pZEG3 vectors, respectively. The *Xma*I–*Xba*I fragments from vectors pZEG1/2/3 were cloned into *Xma*I–*Xba*I sites of the binary vector pTLH221 (2.3), which contains the *bar* gene, as a selection marker. The final GUS-constructs, pCMG1, pCMG2 and pCMG3 and the intermediate vectors were sequenced with primers M13 Reverse, M13 Forward (-20), P1, P4 and P7.

pZE-GFP (*ZmEA1*p::*ZmEA1-GFP*::*NOS*t): this construct contains a ZmEA1:GFP fusion protein under the control of the full-length *ZmEA1* promoter and the NOS terminator from *A. tumefaciens* and was generated in two steps. In the first step, full-length *ZmEA1* cDNA together with its promoter were amplified by a standard PCR from the plasmid pZmEA1-P8-Qb (2.10) with primers ZE-Sac and ZE-Not and cloned using the TOPO TA Cloning® Kit (Invitrogen, Karlsruhe) into the vector pCR®2.1-TOPO® (2.3). After cloning, the plasmid hence obtained was sequenced with primers M13 Reverse, M13 Forward (-20), P1, P4, P7 and RT2. In the second cloning step, the plasmid from the first step was digested with the restriction enzymes *Sac*I and *Not*I and the fragment containing the *ZmEA1* cDNA together with its promoter was cloned into the *Sac*I and *Eag*I sites of the vector pL29GFPeag, generating the final construct pZE-GFP. The vector pL29GFPeag was generated by DNA Cloning Service (Hamburg) by amplifying the *GFP* coding sequence from the vector pMon30049 (2.3) and the *NOS*-terminator from the vector pBi121 (2.3) and cloning both fragments into the vector pLitmus™ 29 (2.3).

pLNU-GFP (*Ubi*p::*GFP*::*NOS*t): this construct contains the *GFP* under the control of the maize ubiquitin promoter and the NOS terminator from *A. tumefaciens* and was generated by DNA Cloning Service (Hamburg). *GFP* coding sequence was amplified from the vector pL29GFPeag (see previous paragraph) with primers M13 Reverse and GFPXho, restricted with *Spe*I and *Xho*I and cloned into the *Spe*I and *Sal*I sites of the vector pLNU-AB (2.3). Sequencing of pLNU-GFP was done using the primers Ubi1 and NosU.

pLG-ZmEA1 (*Ubi*p::*ZmEA1-GFP*::*NOS*t): this construct contains a ZmEA1:GFP fusion protein under the control of the maize ubiquitin promoter and the NOS terminator from *A. tumefaciens* and was generated by DNA Cloning Service (Hamburg). The full-length *ZmEA1* cDNA was amplified from the plasmid pZmEA1-P8-Qb (2.10) with primers EAF-GFP (*Spe*I) and EAR-GFP (*Bam*HI) and ligated into the *Spe*I and *Bam*HI sites of the vector pLNU-GFP (see previous paragraph). After cloning, sequencing of pLG-ZmEA1 was done using the primer Ubi1.

pZE-AS (*Ubi*p::*ZmEA1*-AS::*NOS*t): this construct contains the full-length *ZmEA1* cDNA in anti-sense orientation under the control of the maize ubiquitin promoter and the NOS terminator from *A. tumefaciens* and was generated by DNA Cloning Service

(Hamburg). The *ZmEA1* cDNA was amplified from the plasmid pZmEA1-P8-Qb (2.10) with primers T6Mfe and T6Bam and ligated in anti-sense orientation into the *Bam*HI and *Eco*RI sites of the vector pUbi-AB (2.3). After cloning, pZE-AS was sequenced using the primer Ubi1.

pZE-RNAi (*Ubi*p::*ZmEA1*-AS::*iF2*intron::*ZmEA1*::*OCS*t): this construct contains the the full-length *ZmEA1* cDNA in sense and anti-sense orientation under the control of the maize ubiquitin promoter and the OCS terminator from *Agrobacterium tumefaciens* and was generated by DNA Cloning Service (Hamburg) in two steps. In the first step, *ZmEA1* cDNA was amplified from the plasmid pZmEA1-P8-Qb (2.10) with primers T6Mfe and T6Bam and ligated in anti-sense orientation into the *Bam*HI and *Eco*RI sites of the vector pUbi-iF2 (2.3), generating the intermediary vector pUbi-ZmEA1. In the second step, *ZmEA1* cDNA was again amplified from the plasmid pZmEA1-P8-Qb (2.10) but with primers T6Bsr and T6Mlu and ligated in sense-orientation into the *BsrG*I and *Mlu*I sites of the vector pUbi-ZmEA1, the final pZE-RNAi construct being hence generated. The plasmids pUbi-ZmEA1 and pZE-RNAi were sequenced with primer Ubi1 and OR, respectively.

2.16 Stable transformation of maize and rice

2.16.1 Biolistic transformation of maize

For the generation of stable transformed RNAi-, AS- and GFP-maize plants, immature embryos (10-12 days after pollination) from the maize inbred line A188 and from crosses of the lines A188 and H99 were used. Isolated embryos were cultivated for 1 day with the scutellum opposite to an osmotic medium and then transferred for 7-14 days on N6 induction medium (D'Halluin *et al*., 1992). Embryos were transferred again to osmotic medium for 4 h prior to transformation. Transformation was obtained by bombarding plasmid coated gold particles (0.4-0.8 µm diameter, Heraeus, Karlsruhe) into the developing embryogenic calli using a Biolistic PDS-1000/He device (Bio-Rad, München) with 1350 psi rupture discs under a vacuum of 27 mmHg according to Brettschneider *et al*. (1997). Gold particles were coated with plasmid DNA (2.5 µg DNA of each plasmid) according to Becker *et al*. (1994). The plasmids pZE-RNAi (2.15), pZE-AS (2.15) or pZE-GFP (2.15) were each co-transformed with the plasmid p35S-PAT (2.3), carrying the phosphinothricin acetyl transferase (*PAT*) gene from *Streptomyces*

viridochromogenes for resistance against phosphinothricin (PPT), the active component of the glufosinate ammonium herbicide, as a selectable marker. Bombarded embryos were left on osmotic medium for 20-24 h before going into regeneration and selection processes performed after Brettschneider *et al*. (1997). After transferring into the green house, T0 transgenic plants were selected by spraying two times within two weeks with a solution compound of 200 mg/ml glufosinate ammonium and 0.1% Tween 20. Herbicide selection of T1 transgenic plants was performed by dot spotting directly on the leaves with 5 µl of 1 µg/µl glufosinate ammonium solution.

2.16.2 *Agrobacterium*-mediated transformation of maize and rice

Agrobacterium-mediated transformation method was performed according to the protocol from patent number WO9209696 to transform the plasmids pCMG1, pCMG2 and pCMG3 (2.15) into the rice inbred line M202. The plasmid pCMG1 was transformed as well into the maize He89 derived line using the *Agrobacterium*-mediated transformation method according to the protocol from patent number WO9837212.

2.17 Transient biolistic transformation of onion epidermal cells

Inner peels from onions (4-6 cm diameter) were used for transient biolistic transformation, which was performed after Scott *et al.* (1999) with some modifications. Gold particles (0.4-0.8 µm diameter, Heraeus, Karlsruhe) coated with 10 µg of pLG-ZmEA1 plasmid DNA (2.15) according to Becker *et al*. (1994) were bombarded into the onion epidermal cells using a Biolistic PDS-1000/He device (Bio-Rad, München) with 1100 psi rupture discs under a vacuum of 28 mmHg. Bombarded onion peels were placed concave side facing down on 0.8% agar plates and incubated for 18-22 h at 22°C in continuous light. As a control, the construct pLNU-GFP (2.15) was transiently transformed into onion epidermal cells, using the same conditions. Analysis of GFP expression was performed by fluorescence microscopy (2.20) using an Axiovert 200 microscope (Zeiss, Göttingen).

2.18 Embryo sac cells isolation and *in vitro* pollination assay in maize

Maize embryo sac cells were mechanically isolated with glass needles from ovule tissues treated with a cell wall-degrading enzyme mixture for 20-30 min at room temperature according to Kranz *et al*. (1991). Cells released were transferred to a mannitol droplet (650 mosM/kg H_2O) covered with oil using a fine capillary attached to a CellTram-Oil hand pump (Eppendorf, Hamburg) and analysed by light microscopy using an Axiovert 200 microscope (Zeiss, Göttingen). Documentation was done using a CAMEDIA C-4040 ZOOM digital camera (Olympus, Tokyo, Japan) and images were processed with Adobe Photoshop 5.5 (Adobe Systems Incorporated, U.S.A.) or Micrografx Picture Publisher 7a (Micrografx Incorporated, Richardson, U.S.A.). Isolated egg cells of the embryo sac were frozen afterwards in a droplet (≤1 µl) of mannitol solution (650 mosM/kg H_2O) using liquid nitrogen and stored at -80°C. Some of the maize egg cells used in this work were kindly provided by Dr. Bantin (University of Hamburg).

In vitro pollination assays were performed after Cordts *et al.* (2001) with some modifications. Unfertilised maize ears with 5-10 cm long silks were harvested and after removing of husk leaves were first cut longitudinally and then transversally (without wounding) to obtain segments of about 5-6 cm length. The segments were kept on wet paper in a Petri dish (15 cm diameter) with the ovule containing part of the ear upwards. All silks were orientated towards one direction, cut to the same length and pollinated with freshly harvested pollen from A188 or transgenic maize plants. To avoid drying, the pollinated segments were covered within the Petri dish with wet filter paper. Sections through ovules were isolated from ovaries 24 hours after pollination (hap), which corresponds to approximately 18 h after *in vitro* fertilisation (IVF), and analysed by light microscopy (as described in the previous paragraph) and/or by fluorescence microscopy (2.20), or used for histochemical GUS-assays (2.19).

2.19 Histochemical GUS-assay

β-Glucuronidase (GUS) activity in transgenic maize plants was analysed histochemically (Jefferson *et al*., 1987). Sections through maize ovules (before pollination or more then 24 hours after *in vitro* pollination) were incubated for few hours

to up to three days at 37°C in staining buffer containing X-Gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid CHA-salt) as a substrate. Depending on the experiment, two different GUS-staining buffers were used, both containing the following main composition after Bantin *et al.* (2001): 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.05% X-Gluc, 0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, pH 7.0. Triton X-100 to 0.1% final concentration was added to the GUS-staining buffer used for analysing sections through unpollinated ovules, while mannitol to 650 mosM/kg H_2O was added to the staining buffer used for analysing ovule sections after *in vitro* pollination assays (2.18). Osmolarity of 650 mosM/kg H_2O was used for stability of the maize embryo sac cells (Kranz *et al*., 1991) and Triton X-100 for a better penetration of the GUS-staining solution into the cells. Stained samples were analysed by light microscopy and documented as described before (2.18).

2.20 Fluorescence microscopy

Fluorescence microscopy was performed with an Axiovert 200 microscope (Zeiss, Göttingen) and with a LSM 510 m confocal laser scanning microscope (CLSM, Zeiss, Göttingen). Samples were excited with UV-light produced by a HBO 50/Ac lamp using Zeiss filter set 38 (excitation: BP 470/40 nm, beamsplitter: FT 495, emission: BP 525/50 nm) at the Axiovert microscope and documentation was done as described before (2.18). For fluorescence analysis using the CLSM, samples were excited by 488 nm with an Argon laser and images were taken using the LSM Software, version 3.2 (Zeiss, Göttingen). In both cases, the images obtained were processed as described before (2.18).

3 Results

3.1 Sequencing and cluster analysis of maize egg cell cDNAs

With the aim to identify genes specifically expressed in the unfertilised egg cell of maize, 1056 clones of an egg cell cDNA library (Dresselhaus *et al.*, 1994) were sequenced, generating 1004 ESTs ("expressed sequence tags"). After masking sequence repeats (14 sequences), eliminating sequences smaller then 60 bp (1 sequence) or sequences containing only the 3' terminal poly(A) region (1 sequence), 988 sequences were obtained. Sequences homologous on at least 75 bases and with at least 96% sequence identity over the full-length sequence were clustered, generating 138 clusters and 399 singletons (sequences which cannot be included into a cluster). Using the CAP3 algorithm, clusters were assembled into 136 contigs (corresponding to the consensus sequence obtained after the assembly of many or all sequences of the cluster) and 9 singlets (sequences not homologous enough to be included into a contig). In total, 544 Tentative Unique Genes (TUGs) were obtained, potentially representing 544 different genes. Using BLASTX, these 544 TUGs were compared with the Swissprot and TrEMBL database. 428 TUGs had a valid hit. The results for the largest contigs are shown in Table 3.1.

Table 3.1: 58 largest contigs from a maize egg cell cDNA library (*ZmEC*) and the best hit to the Swissprot and TrEMBL databases.

The number of ESTs and the corresponding best Swissprot and TrEMBL hit are given for each of the contig listed.

***ZmEC* contigs**	**Number of ESTs**	**E-value**	**Homology**
Contig 1.1	46	0	ZmTLA1
Contig 3.1	**32**	**-**	**No hit**
Contig 4.1	27	2,00E-62	Hypothetical protein P0705A05.11
Contig 2.2	23	1,00E-64	ZmES2/3/4 precursor
Contig 2.1	20	1,00E-38	ZmES1 precursor
Contig 5.1	18	-	No hit
Contig 6.1	16	3,00E-68	Hypothetical protein OsJBa0064M23.12
Contig 7.1	16	6,00E-82	Peptidyl-prolyl cis-trans isomerase
Contig 8.1	13	3,00E-88	Eukaryotic translation initiation factor eIF-5A
Contig 9.1	12	2,00E-15	Hypothetical 10.1 kDa protein F5H14.21
Contig 10.1	10	2,00E-29	Putative polyprotein
Contig 11.1	9	3,00E-84	Hypothetical protein OsJNBa0043A12.6
Contig 12.1	9	5,00E-35	Hypothetical protein
Contig 13.1	8	3,00E-88	Eukaryotic translation initiation factor eIF-5A

Table 3.1 (continued)

ZmEC contigs	Number of ESTs	E-value	Homology
Contig 14.1	8	3,00E-45	Hypothetical protein OsJ1081_B12.28
Contig 15.1	8	3,00E-09	Ubiquitin-like protein
Contig 16.1	7	3,00E-11	Hypothetical protein OsJNBb0056B16.13
Contig 17.1	6	5,00E-09	Hypothetical protein OsJNBb0056B16.13
Contig 18.1	6	3,00E-81	Cyclophilin ROC7
Contig 19.1	6	0	Elongation factor 1 alpha
Contig 20.1	5	6,00E-99	40S ribosomal protein S8
Contig 21.1	5	8,00E-80	Calmodulin
Contig 22.1	4	7,00E-59	Hypothetical proteins P0524G08.1
Contig 23.1	4	1,00E-35	At-LS1 product (light-inducible protein-like)
Contig 24.1	4	1,00E-77	Putative GTP-binding protein
Contig 25.1	4	1,00E-44	Putative 60s ribosomal protein L25
Contig 26.1	4	8,00E-46	Ubiquitin-like protein
Contig 27.1	4	7,00E-94	60S ribosomal protein L9
Contig 28.1	4	8,00E-46	Hypothetical protein OsJNBb0006N15.11
Contig 29.1	4	3,00E-73	Hypothetical protein
Contig 30.1	4	2,00E-97	Ribosomal protein L18
Contig 31.1	3	8,00E-40	Hypothetical protein At2g27980
Contig 32.1	3	5,00E-35	Putative NADH dehydrogenase 10.5K chain
Contig 33.1	3	5,00E-52	Calcium-binding protein precursor
Contig 34.1	3	-	No hit
Contig 35.1	3	4,00E-20	Fiber protein Fb11
Contig 36.1	3	6,00E-67	Putative transcription factor
Contig 37.1	3	-	No hit
Contig 38.1	3	2,00E-30	Hypothetical protein OsJNBa0053B21.1
Contig 39.1	3	7,00E-25	60S ribosomal protein L39
Contig 40.1	3	6,00E-40	Putative G-box binding protein
Contig 41.1	3	3,00E-96	60S ribosomal protein L18a
Contig 42.1	3	2,00E-70	Histone H3.3
Contig 43.1	3	8,00E-35	ATP synthase epsilon chain
Contig 44.1	3	9,00E-49	Hypothetical protein OsJNBa0029H02.21
Contig 45.1	3	1,00E-124	Putative esterase D
Contig 46.1	3	7,00E-83	Ubiquitin-conjugating enzyme E2
Contig 47.1	3	-	No hit
Contig 48.1	3	6,00E-27	Putative glucose-6-phosphate/ phosphate-translocator precursor
Contig.49.1	3	-	No hit
Contig 50.1	3	2,00E-33	Putative 40S ribosomal protein S26
Contig 51.1	3	-	No hit
Contig 52.1	3	6,00E-39	Thioredoxin H-type TRX-H
Contig 53.1	3	2,00E-55	Ribosomal protein S10
Contig 54.1	3	3,00E-56	Putative ubiquitin-conjugating enzyme E2
Contig 55.1	3	1,00E-121	20S proteasome subunit alpha-5
Contig 56.1	3	7,00E-30	60S acidic ribosomal protein P3 P1/P2-like
Contig 138.1	3	1,00E-21	Mitochondrial ATP synthase 6 kDa subunit

The results of the BLASTX analysis showed that the second largest contig (contig 3.1) of the maize egg cell cDNA library did not show homology to anything known in public databases, neither genes nor ESTs. Among the most abundant contigs of this cDNA

library, contig 1.1 and contigs 2.1 and 2.2 presented 100% homology to the *ZmTLA1* (*Zea mays Transparent Leaf Arrea1*) and *ZmES1-4* (*Zea mays Embryo Sac1-4*) genes, previously identified and characterised as gametophyte- and female gametophyte-specific genes in maize, respectively, that play a role in cell differentiation and the fertilisation process (Dresselhaus *et al.*, unpublished; Amien, 2003). 24% of the contigs matched to hypothetical proteins, most of them from *Arabidopsis thaliana* (At) and *Oryza sativa* (Os) and 15% were classified as putative proteins. A hit could not be found for 13.8% of the contigs. Interestingly, only 1 contig (2% of the total contigs) showed homology to a histone, indicating that the egg cell is not prepared for fast DNA synthesis. While 8.6% showed homology to proteins involved in protein stabilisation and degradation (Ubiquitin-like proteins, Ubiquitin-conjugating enzymes, 20S proteasome), 20.7% of the contigs showed homology with proteins involved in translation (translation initiation factors, ribosomals proteins, elongation factors), suggesting that although the egg cell is relatively quiescent cell, it may be prepared for rapid increase in metabolic activity after fertilisation.

3.2 Functional analysis of maize egg cell TUGs

Functional analysis has been performed on the maize egg cell 428 TUGs with a valid hit in the Swissprot and TrEMBL databases according to the molecular function category of the Gene Ontology classification (The Gene Ontology Consortium, 2004). The results obtained are illustrated in Fig. 3.1.

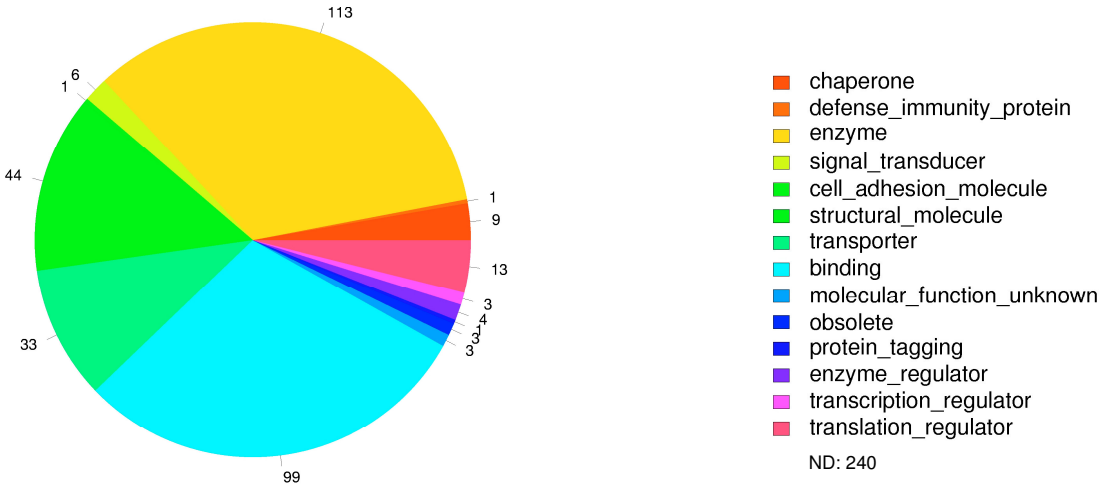

Fig. 3.1: Functional classification of maize egg cell TUGs having a Swissprot_TrEMBL hit in the molecular function category of the Gene Ontology classification. (legend on the next page)

The left part shows a pie chart with *ZmEC* contigs corresponding to molecular function category and the number of TUGs of a specific class. The description of classes is shown on the right. (ND: not determined).

The results of this functional classification analysis showed that a molecular function category could not be addressed to the majority (56%) of TUGs. Of the 428 TUGs analysed, 26% could represent enzymes and 23% are possibly involved in binding reactions - nucleic acid, ion, receptor, protein or peptide binding. 10% TUGs were classified as structural molecules, such as structural constituent of extracellular matrix or cell wall, nuclear pore or ribosome, and 8% represent transporters, for example of ions, lipids, peptides, nitric oxide or boron. 3% TUGs encode translational regulators, 0.7% transcriptional regulators and 0.9% enzyme regulators. 2% TUGs encode chaperons and 1.4% signal transducers.

3.3 Selection and expression of *ZmEA1* gene

Contig 3.1 (32 ESTs) is the second largest contig containing 3.3% of egg cell ESTs. This contig showed no homology to known genes or ESTs in public databases (Table 3.1), but a 100% homology to an unpublished cDNA clone (*Zmec* 135) that was identified by Dr. Simone Cordts, University of Hamburg. According to Northern blot analyses, single cell RT-PCR and *in situ* hybridisation results (Cordts, 2000), *Zmec* 135 was shown to be expressed almost exclusively in the maize egg apparatus, consisting of the egg cell and two synergids. After fertilisation, a weak expression was still detectable in zygotes up to the 2-celled stage (Cordts, 2000). Due to this remarkable expression pattern, this clone was selected for a more detailed expression and functional analyses and renamed as *ZmEA1* (*Zea mays Egg Apparatus1*).

In order to prove absence of *ZmEA1* mRNA in most of the tissues investigated by Northern blot analysis (Cordts, 2000) and in additional maize tissues, a more sensitive expression analysis was performed by applying a multiplex RT-PCR (Fig. 3.2). *ZmEA1* transcripts were exclusively detected in the egg cell and not in the other tissues tested. Thus, the egg apparatus-specific expression of *ZmEA1* gene was verified.

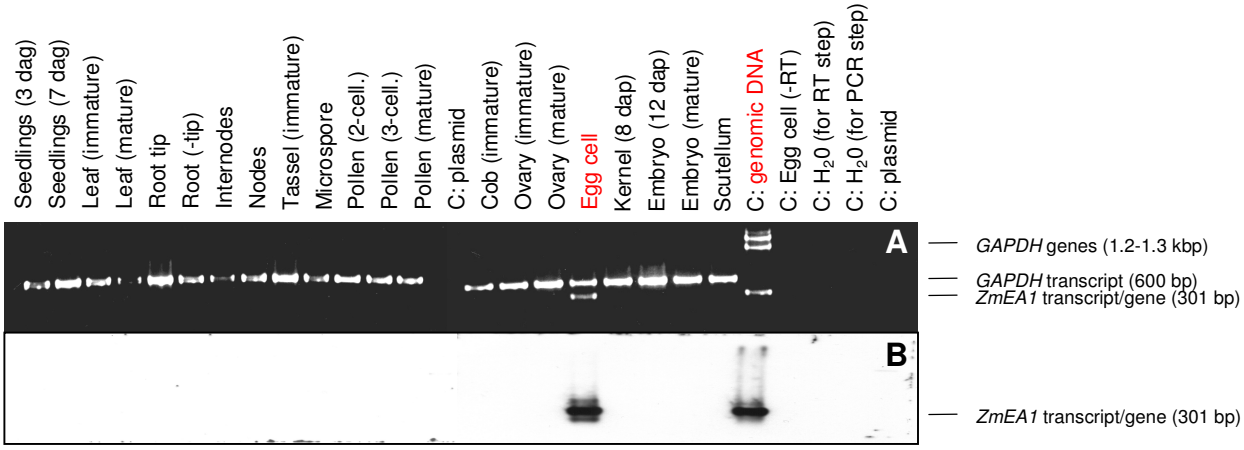

Fig. 3.2: Multiplex RT-PCR detected *ZmEA1* expression exclusively in the egg cell.

(A) *GAPDH* and *ZmEA1* transcripts were amplified performing a multiplex RT-PCR with 1 µg total RNA from maize tissues indicated, using gene-specific primers for *GAPDH* and *ZmEA1*. PCR products were separated in a 1% agarose gel. **(B)** PCR products of (A) were blotted and hybridised with a *ZmEA1*-specific probe. *ZmEA1* transcripts were only detectable in egg cell. PCR products from **egg cell** and **genomic DNA** were used as positive controls for RT and PCR, respectively. 10 pg of *Eco*RI digested 202/4 plasmid has been used as control for Southern blot detection. (C: control, dag: days after germination, dap: days after pollination, *GAPDH*: Glyceraldehyde-3-phosphate-dehydrogenase)

3.4 Structure of the *ZmEA1* gene

3.4.1 Isolation of *ZmEA1* full-length cDNA

ESTs representing the *ZmEA1* gene contained a 3' UTR (untranslated region), which varied in length, due to different poly-adenylation sites used to terminate transcription. ESTs were also incomplete at the 5' end resulting in cDNAs varying from 324 to 447 bp in length. In order to isolate a full-length cDNA of *ZmEA1*, a 5'-RACE was performed with cDNA of maize zygotes. After cloning and sequencing of RACE-products, 98 additional bps were obtained, generating a full-length cDNA of 545 bp.

A reverse Northern blot was performed with maize zygotes cDNA and wheat egg cell cDNA to investigate whether the 545 bp represents the full-length *ZmEA1* cDNA. After gel separation, blotting and hybridisation with a *ZmEA1*-specific probe, a signal was obtained with the maize cDNA, but not with the wheat cDNA (Fig. 3.3). The band detected had a size of around 550 bp, indicating that the 545 bp fragment isolated probably represents the full-length cDNA of *ZmEA1*.

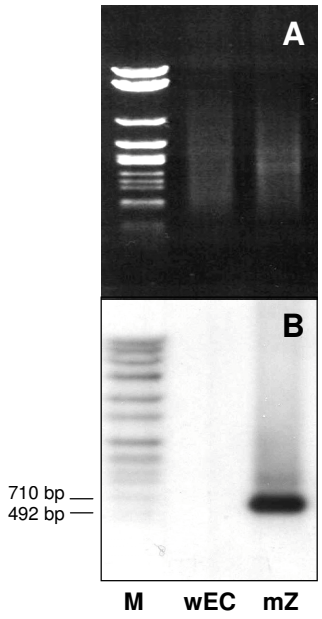

Fig. 3.3: Determination of *ZmEA1* transcript length using a reverse Northern blot.

(A) 100 ng cDNA from maize zygotes (mZ) and from wheat egg cells (wEC) were separated in a 1% agarose gel. **(B)** Reverse Northern blot analysis of (A), hybridised with a *ZmEA1*-specific probe showed a band with the size of about 550 bp in the maize cDNA sample. (M: DNA control "DIG VII-Marker")

3.4.2 Isolation of the *ZmEA1* promoter and transcription start point analysis

The *ZmEA1* promoter was isolated based on a "Genome Walking" technique in two different genome walking rounds using genomic DNA-libraries from maize inbred line A188. The "nested" PCR of the first walking round generated one fragment of about 500 bp and one of about 740 bp from *Dra*I and *Eco*RV libraries, respectively. Both fragments overlapped by 500 bp (100% sequence identity) and included 58 bp of the known cDNA sequence. Thus 682 bp upstream of the so far known sequence could be isolated during the first round. A fragment of about 950 bp containing a 100 bp overlap with the previous isolated upstream sequence was obtained in a "nested" PCR of the second walking round. In total, 1532 bp additional sequence has been isolated after two genome walking rounds.

To identify the transcription start point of the *ZmEA1* gene, standard methods such as Primer Extension Analysis could not be applied as insufficient amounts of RNA can be obtained from maize egg cells or zygotes. Therefore, an alternative strategy has been adopted. After comparison of the *ZmEA1* cDNA with the corresponding genomic sequences, a guanosine-residue (shown in red in Fig. 3.4) was observed at the very 5'-end of the cDNA fragment, which is missing in the genomic fragment. This guanosine-

residue is probably originating from the posttranscriptional “capping” of the *ZmEA1* mRNA. The following adenosine-residue (shown in green in Fig. 3.4), which is located 107 bp upstream of the ATG START codon, was analysed as possible transcription start point (TSP) of the *ZmEA1* gene. Multiplex single cell RT-PCR analyses were performed with maize egg cells using two different forward primers to amplify the *ZmEA1* mRNA. The RT1 primer begins directly with the adenosine-nucleotide representing the possible TSP and the RT2 primer ends with the nucleotide neighbouring the putative TSP (Fig. 3.4). *GAPDH* cDNA was amplified as an internal control. RT1-specific transcripts were obtained with all egg cells tested, while RT2-specific transcripts were never detected (Fig. 3.4). This supports the assumption that either the adenosine-residue or a neighbouring nucleotide is the TSP of the *ZmEA1* gene.

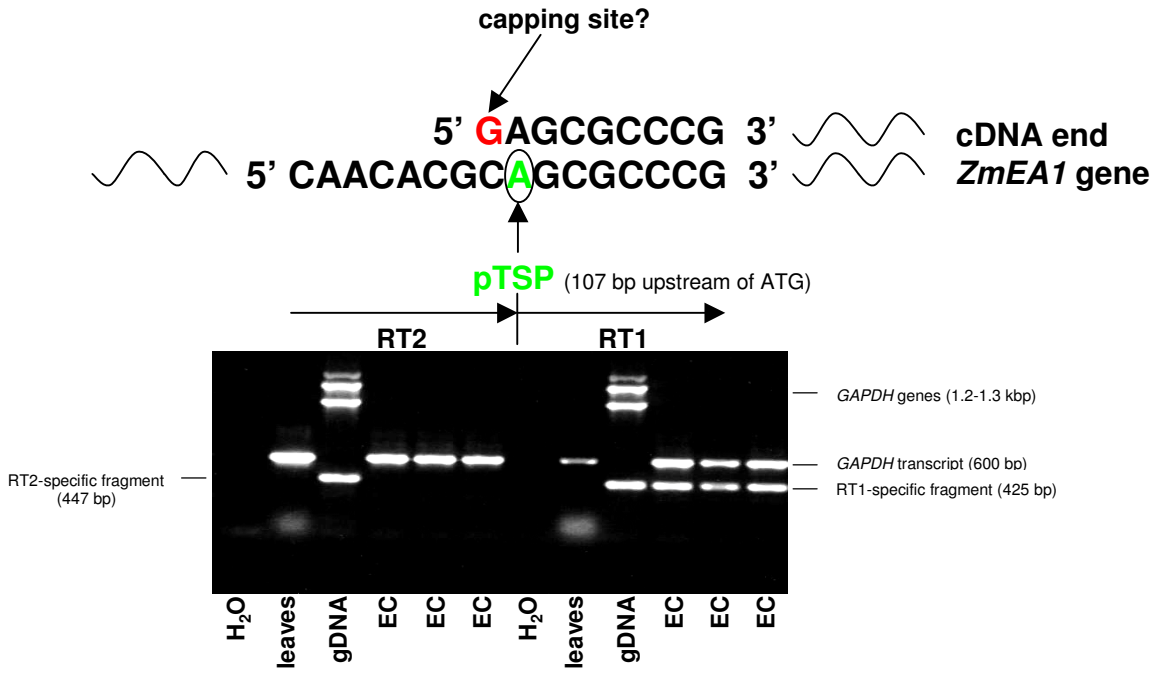

Fig. 3.4: Determination of the *ZmEA1* TSP.

After comparison of *ZmEA1* cDNA and genomic sequences, a guanosine-residue (shown in red) is present at the very 5’-end of the cDNA, but not at the corresponding genome position, probably originating from posttranscriptional “capping” of the *ZmEA1* mRNA. The following adenosine-residue (shown in green), located 107 bp upstream from the ATG START codon, was analysed as the possible TSP. Multiplex single cell RT-PCR analyses were performed with maize egg cells using *GAPDH*-specific primers as well as a *ZmEA1*-specific reverse primer and the forward primers RT1 or RT2, both flanking the putative TSP. RT1-products (425 bp), but not RT2-products (447 bp) were obtained with egg cell samples used, indicating that either the adenosine residue in green is the TSP or one of the neighbouring nucleotides. Maize genomic DNA was used as a positive control and total RNA isolated from maize

leaves, as a negative control. The maize *GAPDH* gene was used as an internal control for the success of the RT-PCR. (EC: egg cell, *GAPDH*: Glyceraldehyde-3-phosphate-dehydrogenase, gDNA: genomic DNA, pTSP: putative transcription start point)

3.4.3 Analysis of *cis*-elements in the *ZmEA1* promoter

The *ZmEA1* promoter sequence (*ZmEA1*p; includes 1463 bp upstream of the transcription start point together with 107 bp 5' UTR of *ZmEA1*) has been searched for homologies using the plant specialised databases PLACE and PlantCARE to identify *cis*-regulatory elements. The results showed homologies to frequent elements involved in binding of basal transcription factors as well as specific transcription factors (Fig. 3.5). The classical TATA box (TATA(T/A)AT, Joshi, 1987) could not be identified some 32±7 nucleotides upstream of the TSP. However, a TACAAAT box was localised -31 to -25 upstream of the *ZmEA1* transcription start point. This motif was identified as a putative TATA-core promoter element in a stamen-specific promoter from rice (Patent number W09213956-A/8). Two CAAT boxes, another basic *cis*-elements common to many eukaryotic promoters, were located 83 and 92 nucleotides upstream of the *ZmEA1* TSP. Additional CAAT-motifs were found over the whole length of the promoter region.

Many more specific regulatory elements were identified (Table 3.2) such as ARF (auxin response factor), "Pyrimidine box" for gibberellic acid induction, and GCC box as an ethylene-responsive element. A second type includes abiotic factors such as SURE2 (sucrose responsive element 2), LTRECORE (core of low temperature responsive element), WUN (wound-responsive element) and ELRE (elicitor responsive element) motifs for sucrose-, cold-, wounding- and fungal elicitor responsiveness, respectively. Putative light-inducible elements, such as GATA-boxes, GAG-motifs and a rbcS general consensus sequence AATCCAA, found in the promoter region of the gene encoding for rbcS (ribulose-1, 5-bisphosphate carboxylase small subunit), also belong to this class and are very common in the *ZmEA1* promoter region. The third type of elements consists of development factors, among which the MSA (M-specific activator)-like element involved in cell cycle regulation, Myb26 and CCAAT-box as Myb-binding sites, and CATGCAT-like or Ry repeat element shown to be involved in seed-specific gene regulation. A SEF3 (soybean embryo factor 3) binding site identified in the 5' UTR of *ZmEA1* can be added to the same third type of *cis*-acting elements. A LTRE motif (ACCGACA; Nordin *et al.*, 1993), a GATA-box, a GAG-motif and two CAAT-boxes have been identified as well in the 3' UTR region of the *ZmEA1* gene (data not shown).

Table 3.2: Putative specific regulatory elements within the *ZmEA1* promoter sequence.

Various regulatory elements in the *ZmEA1* promoter region were identified by sequence analysis in PLACE and PlantCARE databases. For more details, see text.

Category of the induction signal	Motif name	Consensus sequence	Number	Reference
Auxin	ARF binding site	TGTCTC	2	Ulmasov *et al.*, 1997
Gibberellic acid	Pyrimidine box	TTTTTTCC	1	Cercos *et al.*, 1999
Ethylene	GCC box	AGCCGCC	2	Sato *et al.*, 1996
Sucrose	SURE2	AATACTAAT	2	Grierson *et al.*, 1994
Cold	LTRECORE	CCGAC	6	Jiang *et al.*, 1996
Wounding	WUN-motif	TAATTACTA, TAATTTCAT	1 1	Pastuglia *et al.*, 1997
Fungal elicitor	ELRE or W-elements	(T)TGAC(C)	2	Rushton *et al.*, 1996
Light	GATA-box rbcS consensus GAG-motif	GATA AATCCAA AGAGATG	3 2 1	Lam and Chua (1989) Manzara and Gruissem (1988) Argüello-Astorga and Herrera-Estrella (1996)
Cell cycle regulation	MSA-like	aCCAACGGa	1	Ito *et al.*, 1998
Myb binding sites	Myb26 CCAAT-box	GTTAGGTT CAACGG	1 1	Uimari and Strommer, 1997 Wissenbach *et al.*, 1993
Seed-specific gene regulation	RY repeat element SEF3 binding site	CATGCAT AACCCA	1 1	Lessard *et al.*, 1993 Allen *et al.*, 1989

Fig. 3.5 shows the location of putative regulatory elements in the *ZmEA1* promoter sequence.

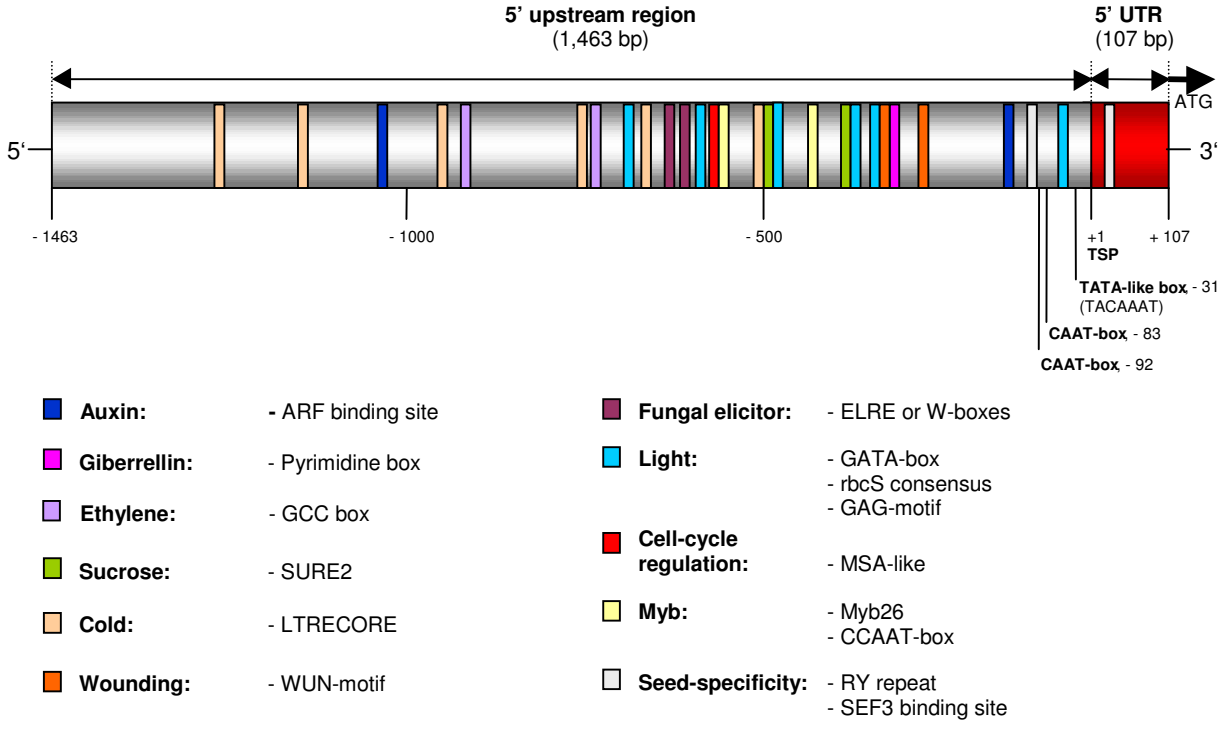

Fig. 3.5: Schematic representation of the *ZmEA1* promoter. (legend continued on the next page)

The figure shows the positions of putative regulatory elements within the *ZmEA1* promoter region. The positions of both TSP and ATG START codon are also shown. A TATA-like box and two CAAT-boxes were identified at positions -31 to -25, -83 to -80 and -92 to -89, respectively, relative to TSP. All specific *cis*-elements are explained in the body of the text. The bold arrow indicates the beginning of translation. (TSP: transcription start point)

The AT content of *ZmEA1* promoter region is relatively high from position -568 to -188 and from -1460 to -1310, upstream of the transcription start point. One direct repeat element, AATACTAATCCAA, was identified in the AT-rich region closer to the TSP at position -498 to -486 and -386 to -374 (Fig. 3.6).

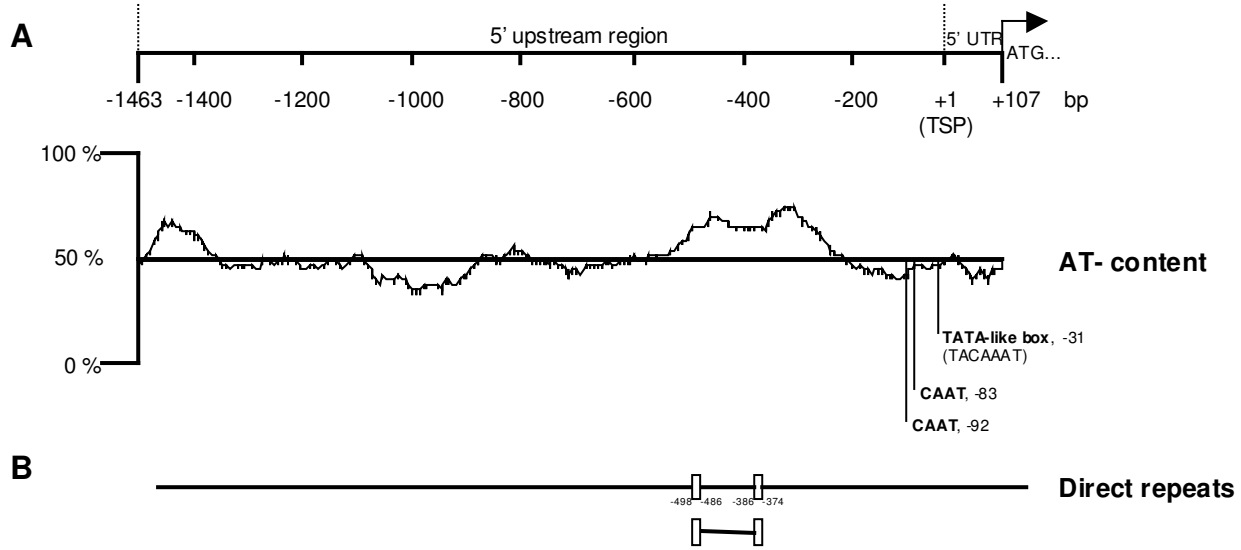

Fig. 3.6: AT-content and sequence repeats in the promoter region of *ZmEA1*.

The *ZmEA1* promoter sequence from position –1463 bp towards +107 bp relative to the transcription start point (TSP) was analysed for AT-content distribution and sequence-repeats.

(A) Distribution of the AT content in the *ZmEA1* promoter. The position of TSP, 5' untranslated region (UTR), ATG START codon and putative CAAT and TATA boxes are shown. The arrow indicates the beginning of translation. **(B)** One direct repeat element with the sequence AATACTAATCCAA is present at positions -498 to -486 and -386 to -374, relative to the TSP.

3.4.4 Intron analysis and genomic structure of *ZmEA1*

With the aim to investigate the presence of introns in the *ZmEA1* gene, a multiplex PCR was performed with genomic DNA from the maize inbred line A188, a maize egg cell cDNA library, cDNA from maize zygotes and wheat egg cells using primers derived from the 5' and 3'-end of the full length *ZmEA1* transcript. Amplification of the *GAPDH* gene

served as an internal control. As shown in Fig. 3.7, signals were obtained with all maize samples, but not with wheat cDNA. The size of *ZmEA1* transcript and genomic fragment obtained was similar. Sequencing of the full length *ZmEA1* gene of 545 bps confirmed this result (Fig. 3.8). The conclusion is that *ZmEA1* represents a gene without introns. In contrast, amplification of partial *GAPDH* genes indicates the presence of intron(s) of a total length of at least 600 bp.

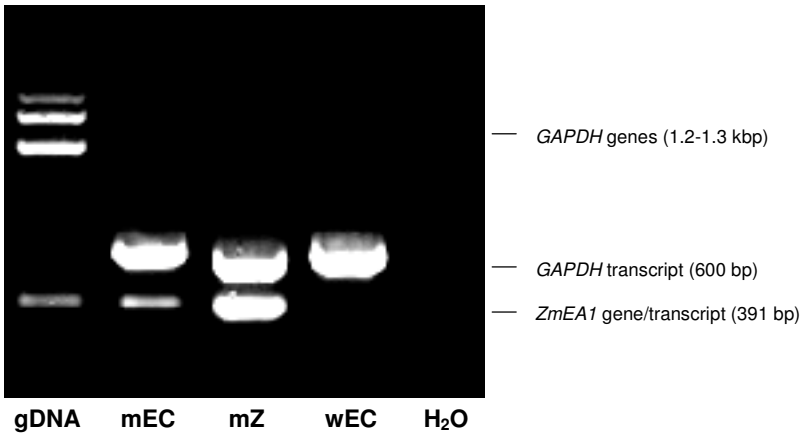

Fig. 3.7: Multiplex PCR analysis showing that *ZmEA1* represents a gene without introns.
Products of a multiplex PCR performed with different cDNA populations using *ZmEA1*-specific and *GAPDH*-specific primers were separated in a 1% agarose gel. Specific transcripts for *GAPDH* (600 bp) and *ZmEA1* (391 bp), as well as specific genomic fragments of *GAPDH* (1.2-1.3 kb) and *ZmEA1* (391 bp) can be distinguished. The maize *GAPDH* was used as an internal control for the success of the PCR. (*GAPDH*: Glyceraldehyde-3-phosphate-dehydrogenase, gDNA: genomic DNA isolated from maize leaves, H_2O: water control, mEC: cDNA library of maize egg cells, mZ: cDNA of maize zygotes, wEC: cDNA of wheat egg cells)

The genomic structure of *ZmEA1* is summarised in Fig. 3.8. The length of the isolated promoter region is 1463 bp (position -1463 to -1) and the single exon is 545 bp, with a 5' UTR of 107 bp (position +1 to +107) and a 3' UTR of 153 bp (position +393 to +545), calculated from the putative start point of transcription at position +1. The open reading frame of *ZmEA1* (position +108 to +392, relative to transcription start point) is GC-rich (%G+C= 65.26) and encodes a small protein of 94 amino acids (Fig. 3.8).

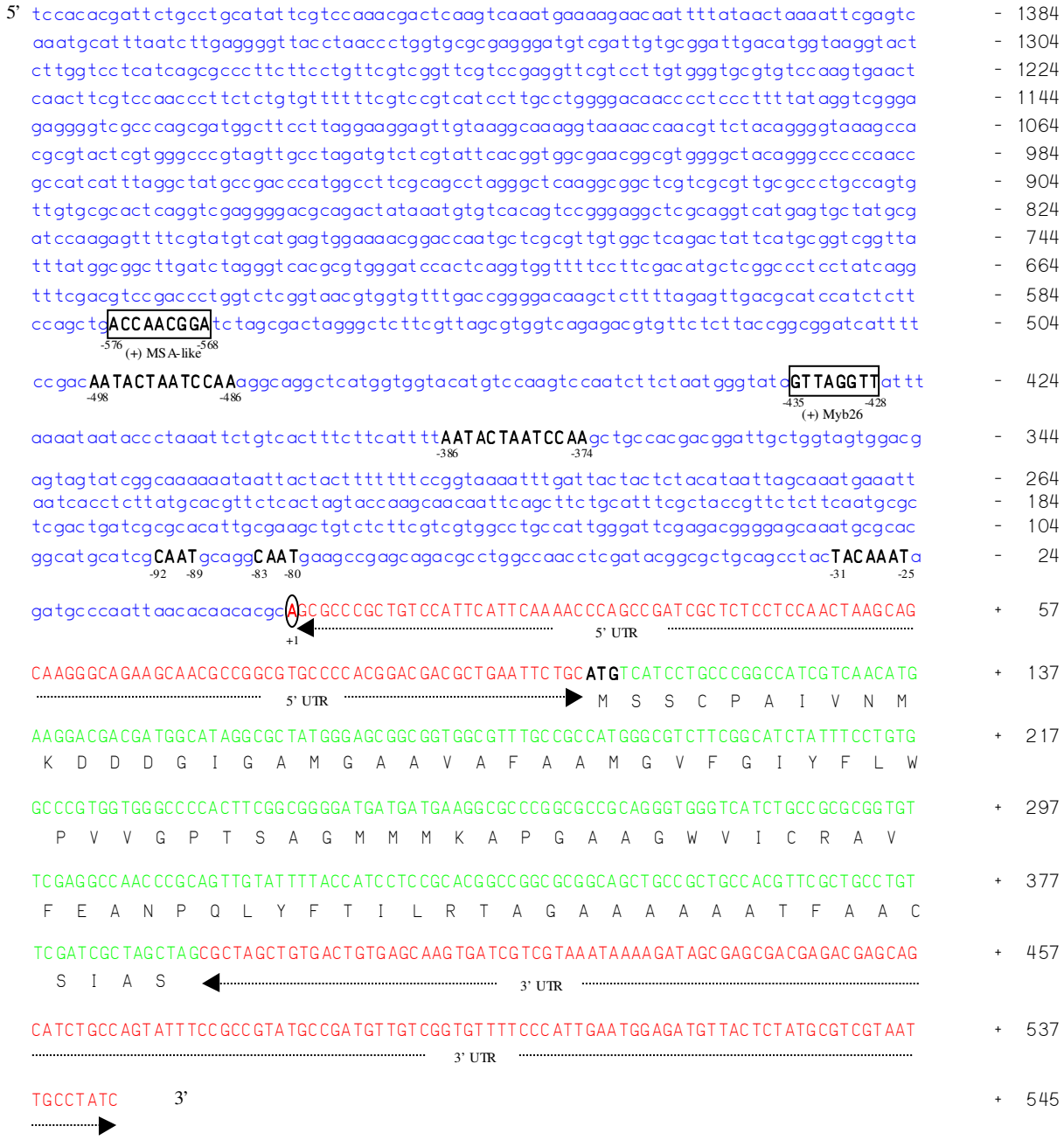

Fig. 3.8: Genomic structure of *ZmEA1*.

The *ZmEA1* promoter (1463 bp) is shown in blue, the 5' UTR (107 bp) and the 3' UTR (153 bp) are indicated in red and the open reading frame (ORF) of 285 bp DNA sequence is shown in green. The encoded amino acid sequence (94 aa) is shown below the ORF. The putative TATA-like box, two CAAT-boxes, the direct repeat element AATACTAATCCAA and the ATG start codon are shown in bold-black. The putative transcription start point (+1) is encircled. The MSA-like element for cell cycle regulation and the Myb26 binding site are boxed. (+) indicates nucleotide sequence homologous to consensus sequences.

3.4.5 Genomic mapping of *ZmEA1*

In order to study the chromosomal location of *ZmEA1*, the gene was mapped using maize recombinant inbred families CM37 x T232 and CO159 x TX303 (Burr and Burr, 1991). In a first step, good RFLPs (restriction fragment length polymorphisms) using a *ZmEA1*-specific probe were identified between parents of the two mapping populations by Southern blot and hybridisation analyses. The resulting polymorphisms (*Bgl*II for CM37 x T232 (Fig. 3.9) and *Pst*I for CO159 x TX303 (data not shown)) were scored and placed on the Brookhaven National Laboratory map using the Map-Maker program (Burr and Burr, 1991). The *ZmEA1* locus maps to the long arm of chromosome 7 between the molecular markers *isc* (*b32B*) at position 119.3 and *bnl8.39* at position 124.8, a region that is lacking known phenotypic markers.

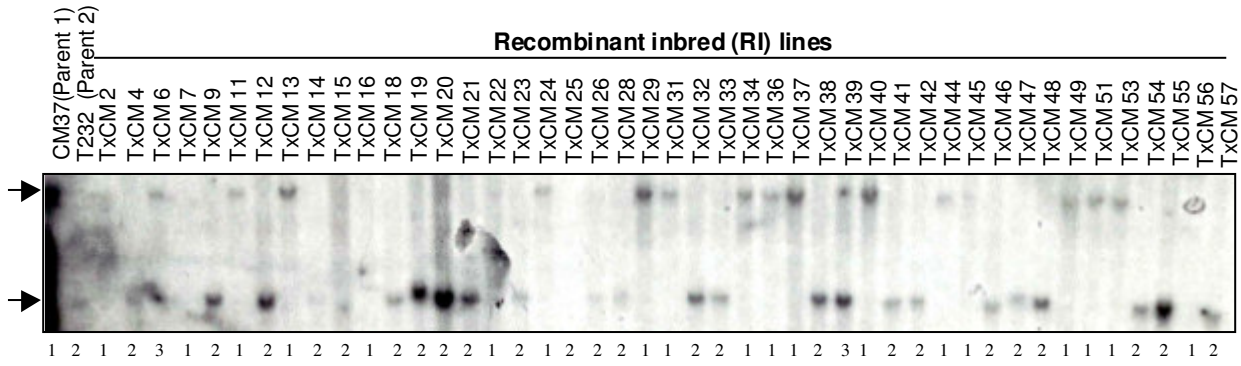

Fig. 3.9: RFLP of *ZmEA1* with the maize recombinant inbred family CM37 x T232.

10 µg DNA from each plant of the RI family CM37 x T232 was restricted with the restriction enzyme *Bgl*II and hybridised with a *ZmEA1*-specific hybridisation probe. Signals similar to parent 1 (CM37) were scored as 1 and those to parent 2 (T232) as 2. A 3 was scored for the occurrence of both parental signals.

3.4.6 Homologous genes of *ZmEA1* in other plant species

To investigate the occurrence of *ZmEA1* homologous genes, comparable genome amounts from maize, *Tripsacum*, rice, barley, *Arabidopsis* and tobacco have been restricted with *Eco*RI and hybridised with a *ZmEA1*-specific probe. Signals were detected in all cereals analysed but not in the dicotyledonous plant species (Fig. 3.10). The signal intensity was decreasing from maize to barley indicating that the homology with *ZmEA1* gene in other cereals is less compared to maize. *ZmEA1* is present as a single copy gene in the maize inbred populations used to map the gene (Fig. 3.9) and in

the maize inbred lines A188, H99 and B73, as well as in *Tripsacum* and in barley (Fig. 3.10). Interestingly, two distinct bands were detected in rice, so that *ZmEA1* seems to have two homologous genes in the rice genome. The occurrence of faint bands observed in all cereals indicates the presence of related, but less homologous genes. Homologous genes in *Arabidopsis* and tobacco could neither be detected under stringent (68°C, 0.2X SSC- final wash; Fig. 3.10) nor with low stringent hybridisation conditions (60°C, 0.5X SSC- final wash; data not shown), even after three weeks exposure of the filters (data not shown).

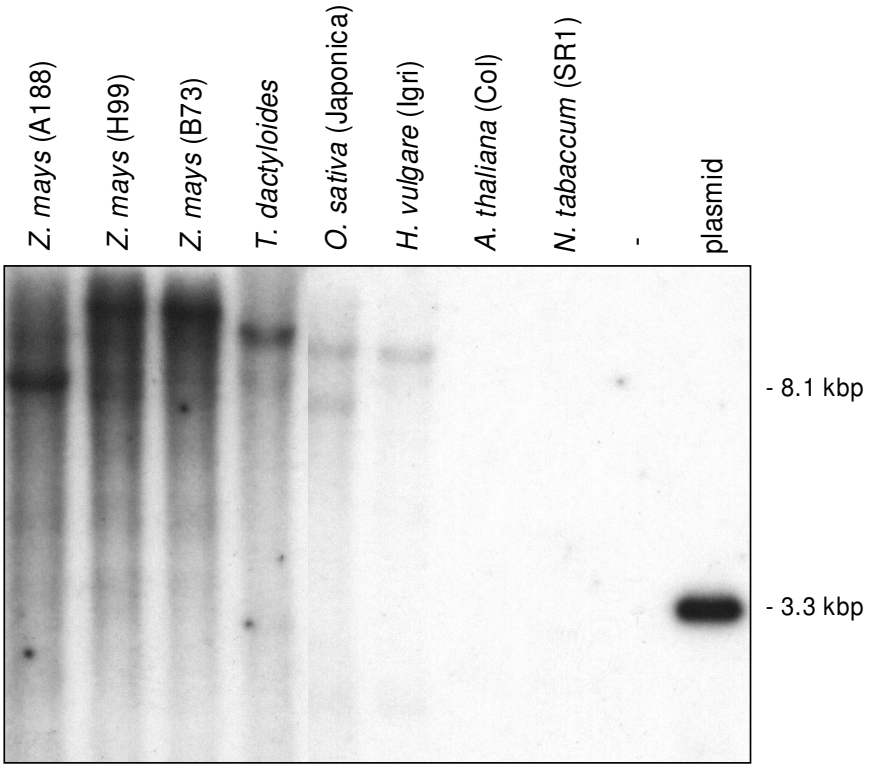

Fig. 3.10: Genomic Southern blot analysis to investigate the occurrence of *ZmEA1* homologous genes in other cereals and dicotyledonous plant species.

Comparable genome amounts (10 to 20 µg genomic DNA) from four cereals and two dicotyledonous plant species were restricted with *Eco*RI, separated in a 0.8% agarose gel, blotted and hybridised with a *ZmEA1*-specific probe under stringent conditions (68°C, 0.2X SSC- final wash). 10 pg of the plasmid p202/4 was digested with *Eco*RI and used as a positive control for the Southern blot analysis. ("-": blank lane)

3.5 Analysis of *ZmEA1* promoter activity in maize and rice

The functionality and activity of the *ZmEA1* promoter was analysed in both maize and rice. Stably transformed transgenic plants containing the *GUS* coding sequence under the control of *ZmEA1* promoter deletion fragments were analysed by histochemical GUS-assays or by RT-PCR, respectively.

3.5.1 Analysis of *ZmEA1* promoter activity in transgenic maize

Transgenic maize was generated by *Agrobacterium*-mediated transformation using the He89 derived line and the construct pCMG1 (2.15) containing the *GUS* gene under the control of the *ZmEA1* full-length promoter (*ZmEA1*p::*GUS*::*NOS*t). The *bar* gene giving resistance to glufosinate ammonium was used as a selection marker. Eleven transformed plants surviving the herbicide selection were further analysed by genomic Southern blot analysis to verify successful integration of the transgene. Genomic DNA was isolated from leaves samples and digested with the restriction endonucleases *Cfr*9I and *Xba*I, which cut the 5´-end of the *ZmEA1* promoter and the 3´-end of the *NOS* terminator, respectively, generating a band of 3,674 bp corresponding to the *Cfr*9I-*Xba*I fragment (Fig. 3.11 B) of plasmid pCMG1. Full-length integration of the transgene was confirmed for six independent transgenic lines (01504, 01804, 02603, 03504, 06203, 07604), each containing the *Cfr*9I-*Xba*I fragment of the expected size (3,674 bp) (Fig. 3.11 A). Lines 01704 and 07404 contained incomplete fragments of the construct. The copy number of the integrations varied from ≥1 to ≥3. Lines 08003 and 08204 did not show any integrations of the pCMG1 construct. The endogenous *ZmEA1*-specific fragment from maize He89 derived line was detected in almost all transgenic plants analysed.

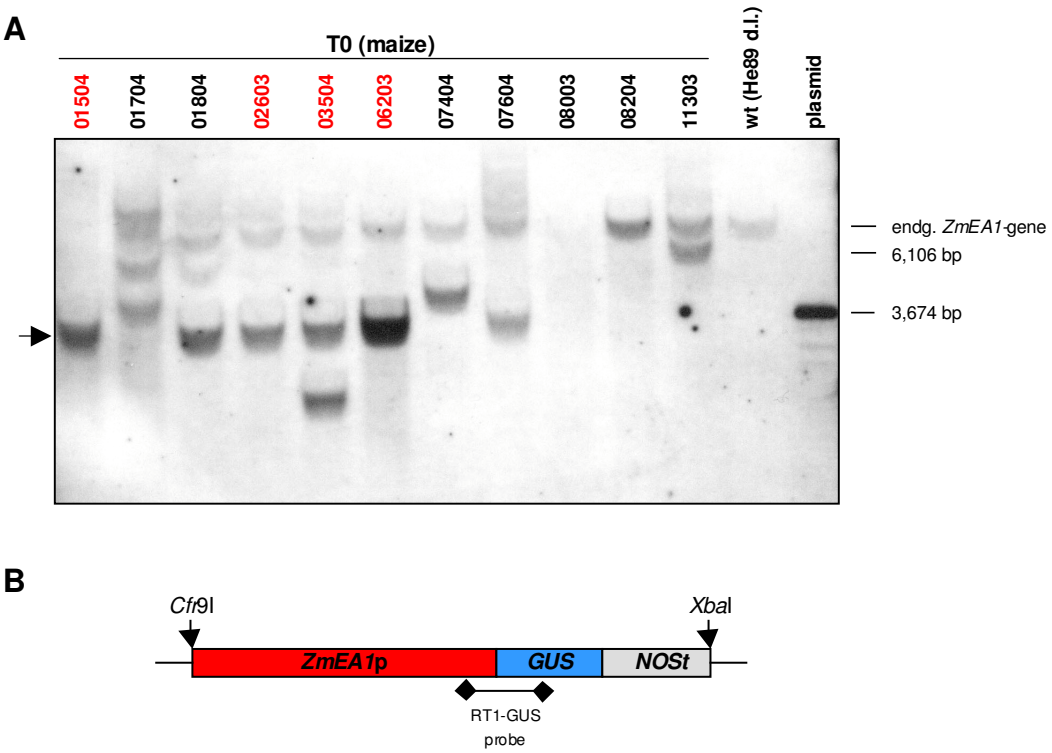

Fig. 3.11: Genomic Southern blot of maize plants transformed with *ZmEA1*p::*GUS*::*NOS*t (pCMG1). **(A)** Eleven maize lines of the T0-generation transformed with pCMG1 were analysed for full-length integration of the construct. Genomic DNA (20-25 µg) was restricted with *Cfr*9I and *Xba*I, separated in a 0.8% gel, blotted and hybridised with a RT1-GUS probe. The band with the size of 3,674 bp (arrow) represents the full-length integration of the transgene corresponding to the *Cfr*9I-*Xba*I fragment **(B)** of plasmid pCMG1. 10 pg of the plasmid pCMG1 restricted with *Cfr*9I and *Xba*I was used as a positive control. Genomic DNA from maize He89 derived line restricted with *Cfr*9I and *Xba*I was used as a negative control and to show the endogenous *ZmEA1* gene. The four different transgenic lines, which were further analysed, are marked in red. (d.l.: derived line, endg.: endogenous).

Histochemical GUS-assays were performed with the four T0-lines 01504, 02603, 03504 and 06203. Maize ovules were excised from ovaries and incubated for 24 h at 37 °C in GUS staining buffer. *GUS*-expression was detected in lines 02603, 03504 and 06203, but not in line 01504. As shown in Fig. 3.12 A, marker gene expression was exclusively detected in the egg-apparatus 24h after staining initiation, demonstrating the functionality of the promoter. A GUS activity could not be detected in other parts of the maize ovule or in leaves, even after more then 3 days in staining solution. Homozygous maize lines expressing *GUS* under the control of the actin promoter (Brettschneider *et al.,* 1997) were used as positive control and wild type maize plants of He89 derived line and A188 line, served as negative controls, respectively. Compared to *ZmEA1* promoter activity, a much stronger GUS activity was observed when driven by the constitutive

actin promoter of rice. After only five hours in staining solution, strong GUS activity was observed in the female gametophyte, especially in the egg apparatus and antipodals, indicating a high expression of actin in these cells (Fig. 3.12 B). The actin promoter seemed less active in the maternal tissues as 24 h incubation was necessary for all ovule cells to be stained. No GUS activity was observed in wild type samples.

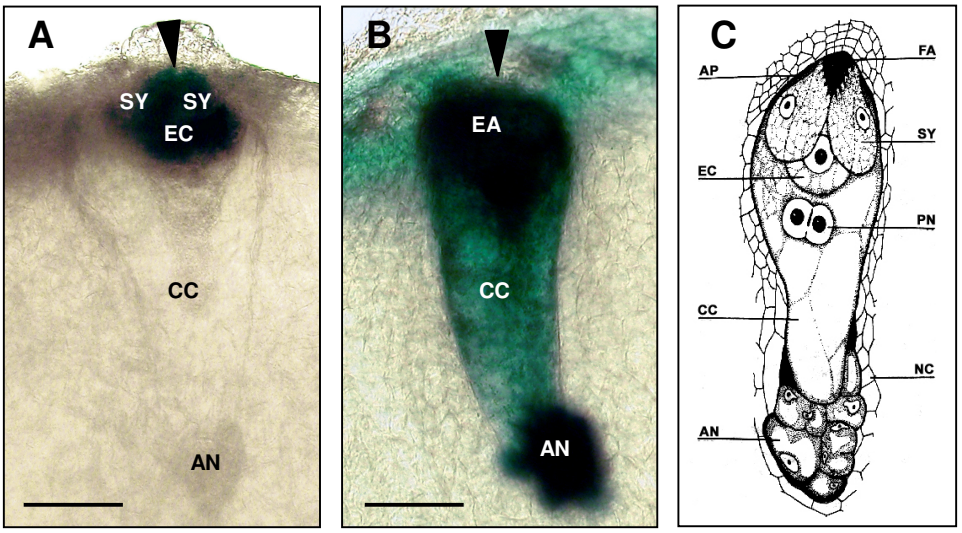

Fig. 3.12: The promoter of *ZmEA1* drives GUS-expression in the egg apparatus of maize.
(A) The *ZmEA1* promoter (1463 bp upstream of the transcription start point together with 107 bp 5' UTR of *ZmEA1)* was used to drive glucuronidase (GUS) in transgenic maize plants. After 24 h in GUS staining buffer, signals were observed exclusively in the three cells of the egg apparatus demonstrating functionality of the *ZmEA1* promoter. Integuments and ovary tissue have been removed. **(B)** Transgenic *Actin*p::*GUS* maize plants were used to investigate actin promoter activity in the female gametophyte before fertilisation. After 5 h in staining solution, signals were observed in all cells of the embryo sac. After 24 h in staining solution, all surrounding maternal tissues have been stained too (not shown), indicating strong requirement of actin filaments in the embryo sac. **(C)** Schematic representation of the female gametophyte from maize, after Diboll and Larson (1966). (AN: antipodals, AP: apical pocket, EA: egg apparatus, EC: egg cell, CC: central cell, FA: filiform apparatus, NC: nucellus cells, PN: polar nuclei, SY: synergids. Open arrow heads mark the surface of the micropyle and closed arrow heads show the five to six layers of nucellar cell files of the micropyle. Bars: 100 µm.).

3.5.2 Analysis of *ZmEA1* promoter activity in rice

Three GUS promoter deletion constructs, pCMG1, pCMG2 and pCMG3 (2.15), respectively, were transformed into the rice inbred line M202 by *Agrobacterium*-mediated transformation. The *bar* gene was used as a selection marker. Rice plants surviving the glufosinate ammonium herbicide selection were analysed by genomic Southern blots and PCR analyses to verify successful integration of the transgene (data not shown). Lines containing full-length copy integration of the transgene were obtained with all constructs. Histochemical analyses with rice ovules have been performed using various methods. However, no significant GUS activities have been observed.

Finally, positive lines with the full-length promoter were examined via mRNA analyses in order to study *ZmEA1* promoter functionality in rice. RT-PCR analyses were performed with the three different transgenic lines 2201, 2301 and 2501 containing full-length integrations of the longest construct pCMG1 (*ZmEA1*p::*GUS*::*NOS*t). cDNAs were generated for each line from poly(A)$^+$ mRNA each extracted from 10 pistils. Two separate standard PCRs were performed with each cDNA sample, using pCMG1-specific primers and *GAPDH*-specific primers. Blots with PCR products were hybridised with *GUS*- and *GAPDH*-specific probes. GUS transcripts (532 bp) were detected for all three transgenic lines showing that *ZmEA1* promoter is also functional in rice (Fig. 3.13 A). GUS expression level for line 2501 was lower compared to the other two lines. This correlates with the result obtained from the genomic Southern blot, showing that the line 2501 contains only one copy integration of the transgene, while the other two lines contain two copies (data not shown). GAPDH-specific transcripts (622 bp) were obtained with all cDNAs used, indicating that the RT step was successful for each sample and that no genomic DNA was present in the sample (Fig. 3.13 B).

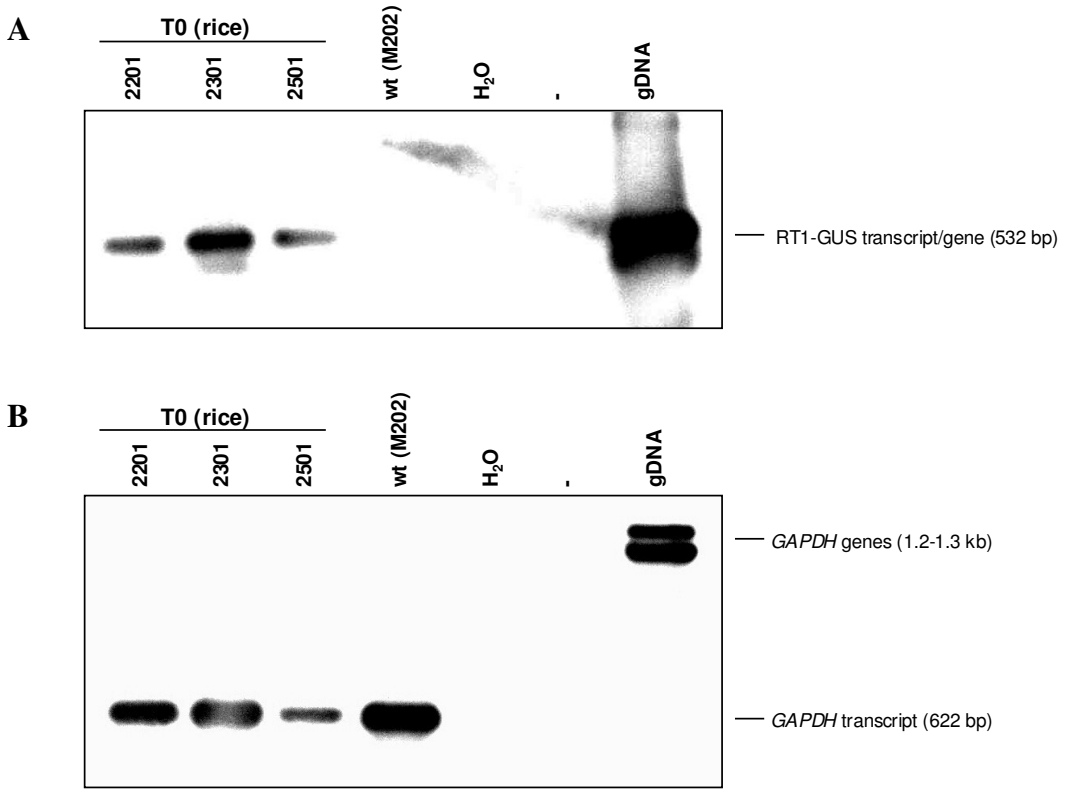

Fig. 3.13: *ZmEA1* promoter activity in rice analysed by RT-PCR.

(A) and **(B)** show Southern blots with PCR products obtained using cDNAs from pistils of three different rice transgenic lines containing the construct pCMG1 (*ZmEA1*p::*GUS*::*NOS*t). pCMG1-specific primers (RT1 and GUS rev) were used for each cDNA in one reaction and *GAPDH*-specific primers (GAPDH-japonica and GAPDH-O.s.2) in a second reaction. A *GUS* specific probe was used to detect pCMG1-specific products in (A) and a *GAPDH*-specific probe to detect *GAPDH* rice-specific products in (B). The maize *GAPDH* gene was used as standard for the success of the RT step. Genomic DNA isolated from leaves of wild type rice line M202 was used as a positive control. (*GAPDH*: Glyceraldehyde-3-phosphate-dehydrogenase, H_2O: water control, "-": blank lane).

3.6 ZmEA1 protein analysis

3.6.1 Protein structure

ZmEA1 amino acid sequence data were analysed using different programs and databases. The results are shown in Fig. 3.14 and Fig. 3.15. *ZmEA1* encodes a small protein of 94 aa (9.5 kDa, 6.2 isoelectric point) with a high degree of hydrophobic residues (50% of amino acids) and a predicted transmembrane domain from position 17/19 to 36/39, depending on the prediction program (Fig. 3.14 A). The PSIpred predicted secondary structure comprises three α-helices and four β-sheets (Fig. 3.14 B). Conserved domains or signal peptides could not be identified. Numerous putative

cleavage sites for enzymes such as proteinases, endopeptidases, pepsin, thermolysin, trypsin, clostripain, etc., were identified in the ZmEA1 protein sequence using the PeptideCutter program. Restriction sites for enzymes such as caspases, enterokinase, hydroxylamine, thrombin, etc. were not predicted for the ZmEA1 protein (data not shown).

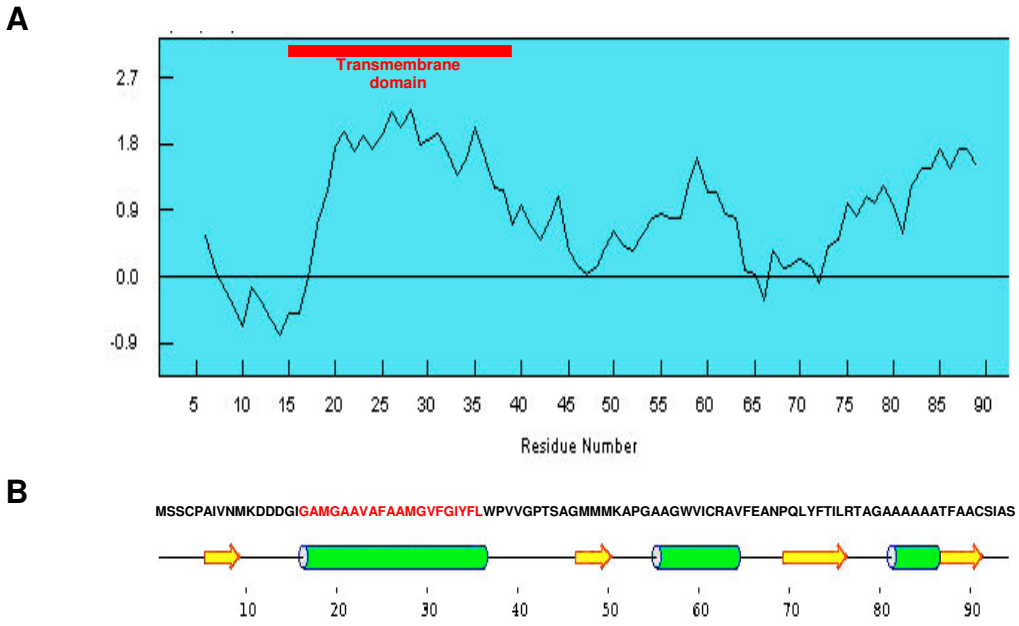

Fig. 3.14: Hydropathy and secondary structure predictions of ZmEA1.

(A) A hydropathy plot shows a predicted transmembrane domain between sequence positions 17 and 36, represented in red. **(B)** Secondary structure prediction showed that the ZmEA1 protein consist of three α-helices and four β-sheets. The amino acid composition of ZmEA1 protein is indicated and the transmembrane domain is written in red.

Corroborating the hybridisation results from Fig. 3.10, two homologous proteins (OsEAL1 and OsEAL2: *Oryza sativa* EA-like) were identified in the rice genome sequence database. The highest homology with ZmEA1 was detected in the C-terminal part of the proteins. Both homologous genes encode small proteins of 113 aa with predicted transmembrane domains (boxed in Fig. 3.15) and are located on the long arm of rice chromosome 7 (BAC83883.1 and BAC83885.1) with three kbp separated from each other, probably due to a recent gene duplication. Interestingly, *ZmEA1* was also mapped to the long arm of chromosome 7 in maize, a region lacking phenotypic markers. While OsEAL1 represents a hypothetical protein, OsEAL2 is weakly expressed, as five ESTs have been identified in callus, young leaf, root and panicle, respectively (EST database at NCBI).

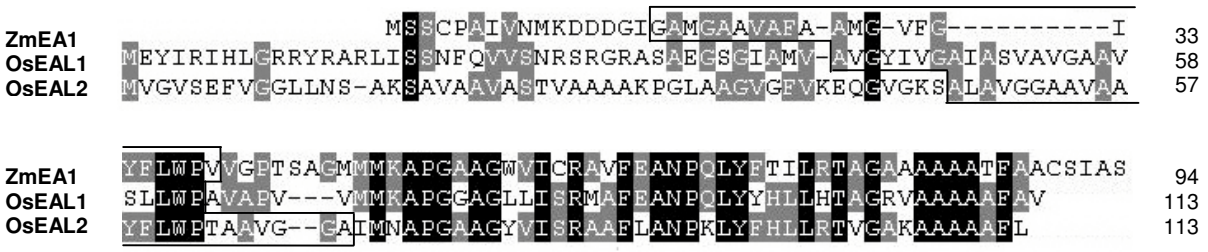

Fig. 3.15: Homologies between maize ZmEA1 and two related rice proteins.

ZmEA1 encodes an oligopeptide of 94 aa with a predicted transmembrane domain (boxed). Two homologous proteins identified in the rice genome (OsEAL1/2, *Oryza sativa* EA-like 1/2) are small proteins of 113 aa with predicted transmembrane domains (boxed). Identical aa, mainly within the C-terminal half of the proteins, are shadowed.

3.6.2 Localisation of ZmEA1 *in vivo*

With the aim to study ZmEA1 protein localisation, the functional full-length *ZmEA1* promoter was used to express a ZmEA1:GFP fusion protein, as immunolocalisation of small proteins/peptides in the female gametophyte of cereals is not possible up to date. Seven independent transformation experiments were conducted with 626 immature embryos from crosses between the maize inbred lines A188 and H99, which were bombarded with the plasmid pZE-GFP (*ZmEA1*p::*ZmEA1-GFP*::*NOS*t). The plasmid p35S-PAT carrying the *pat* marker gene for glufosinate ammonium selection was co-transformed with the GFP construct. Plants surviving the herbicide selection were first analysed by genomic Southern blots for full-length integration of the transgene. Genomic DNA was isolated from leave material and restricted with *Sac*I and *Xho*I generating full-length promoter::GFP-fragments, as shown in Fig. 3.16. Separated and blotted DNAs were hybridised with a GFP-specific probe (Fig. 3.16). An overview of the obtained transgenic lines is shown in Table 3.3.

Table. 3.3: Transformation and regeneration of maize plants containing the *ZmEA1*p::*ZmEA1-GFP* construct.

Genotype of bombarded embryos	Number of experiments	Number of bombarded embryos	Number of regenerated lines	Number of transgenic lines*	Number of transgenic lines* (independent clonal lines) with full-length integration(s) of the transgene
A188xH99	7	626	17	14	13 (9)

* *ZmEA1* transgenic lines

According to the results from Table 3.3, fourteen T0 *ZmEA1* transgenic lines were obtained using the pZE-GFP construct. Thus, transformation efficiency was 2.2%. Full-length integration of the GFP-fusion protein cassette was confirmed for 9 independent transgenic lines, all containing a band of 3,049 bp, corresponding to the fragment *Sac*I-*Xho*I (Fig. 3.16 C) of plasmid pZE-GFP. Line 16.1 contained incomplete fragments of the construct. Some of the lines are clonal (lines 6 and 7, lines 9.1 and 9.2 and lines 8 and 14 in Fig. 3.16 A, as well as lines 27 and 28, data not shown). The copy number of the integrations varied from 2 to 16. Lines 5, 15 and 24 did not show any integrations of the pZE-GFP construct.

Maize GFP-lines 1, 4.1, 6, 9.1 and 9.2 were further analysed in the T1-generation, after T0-plants of these lines were self-pollinated. T1-progenies surviving the herbicide selection were first analysed by genomic Southern blots for the integration of the transgene as shown in Fig. 3.16 B for line 4.1, as an example. Three of eight T1-progenies contained the full-length integration of the transgene and showed the same integration pattern as the parent indicating that transgenes are co-localised at the same chromosomal region.

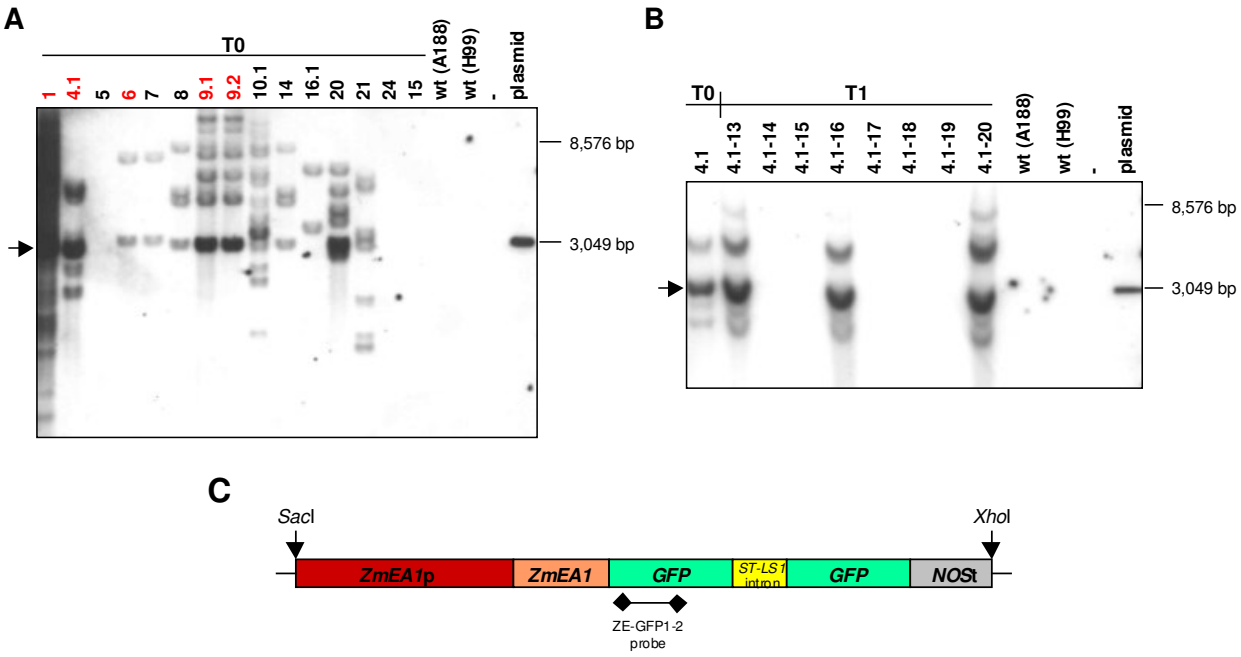

Fig. 3.16: Genomic Southern blots of maize plants transformed with *ZmEA1*p::*ZmEA1-GFP*::*NOS*t (pZE-GFP).

(A) and **(B)** show Southern blots of plants from the T0-generation and T1-generation, containing the pZE-GFP construct. Genomic DNA (20-25 µg) from leaves of these maize plants was restricted with *Sac*I and *Xho*I, separated in 0.8 % agarose gels, blotted and hybridised against a GFP-specific probe. A band of

3,049 bp (arrow) represents the full-length integration of the GFP-fusion protein cassette corresponding to the fragment *Sac*I-*Xho*I **(C)** of plasmid pZE-GFP. 10 pg of plasmid pZE-GFP restricted with *Sac*I and *Xho*I was used as a positive control. Genomic DNAs from maize inbred lines A188 and H99 restricted with *Sac*I and *Xho*I served as negative controls. Five different transgenic lines in (A) are marked in red. These lines were further analysed in the T1-generation. ("-": blank lane, wt: wild type).

Sections through ovules from T0-plants 27 and 28 and from T1-progenies of lines 1, 4.1, 6, 9.1 and 9.2 were analysed using light and fluorescence microscopy to study the localisation of ZmEA1:GFP fusion protein in maize. GFP fluorescence could not be detected in ovule sections from T0-plants 27 and 28, while samples from T1-progenies of all lines analysed showed fluorescence signals within the egg apparatus and nucellus cells below the micropyle. The fusion protein was first detectable in the filiform apparatus and the cell wall material of the egg apparatus in unfertilised young female gametophytes (FGs) (Fig. 3.17 A), shortly after silk emergence. At that stage, faint GFP signals were also visible within the nucellus cell layers at the micropylar region. After silk elongation (>10 cm), a larger and stronger GFP signal of 40-50 µm was detected within the nucellar region of the micropyle of unfertilised mature FGs generating a gradient from the filiform apparatus towards the surface of the micropyle (Fig. 3.17 D and F). A more detailed analysis by confocal laser scanning microscopy (CLSM) was applied to visualise the fusion protein at the micropylar region. ZmEA1:GFP fusion protein was located in the cell walls of nucellus cells below the micropyle with highest concentrations in the cell wall material of the egg apparatus (Fig. 3.17 H and I). Fusion protein accumulated at the surface of nucellus cells at the micropylar region within an area of around 150 µm, but not at the surface of the inner integument. Signals of ZmEA1:GFP fusion protein were no longer detectable in the samples analysed 24 h after *in vitro* pollination (around 18 h after fertilisation). Figure 3.17 J shows a faint reminiscent signal within the filiform apparatus and low autofluorescence of the degenerated and receptive synergid. The pollen tube is still visible (Fig. 3.17 K).

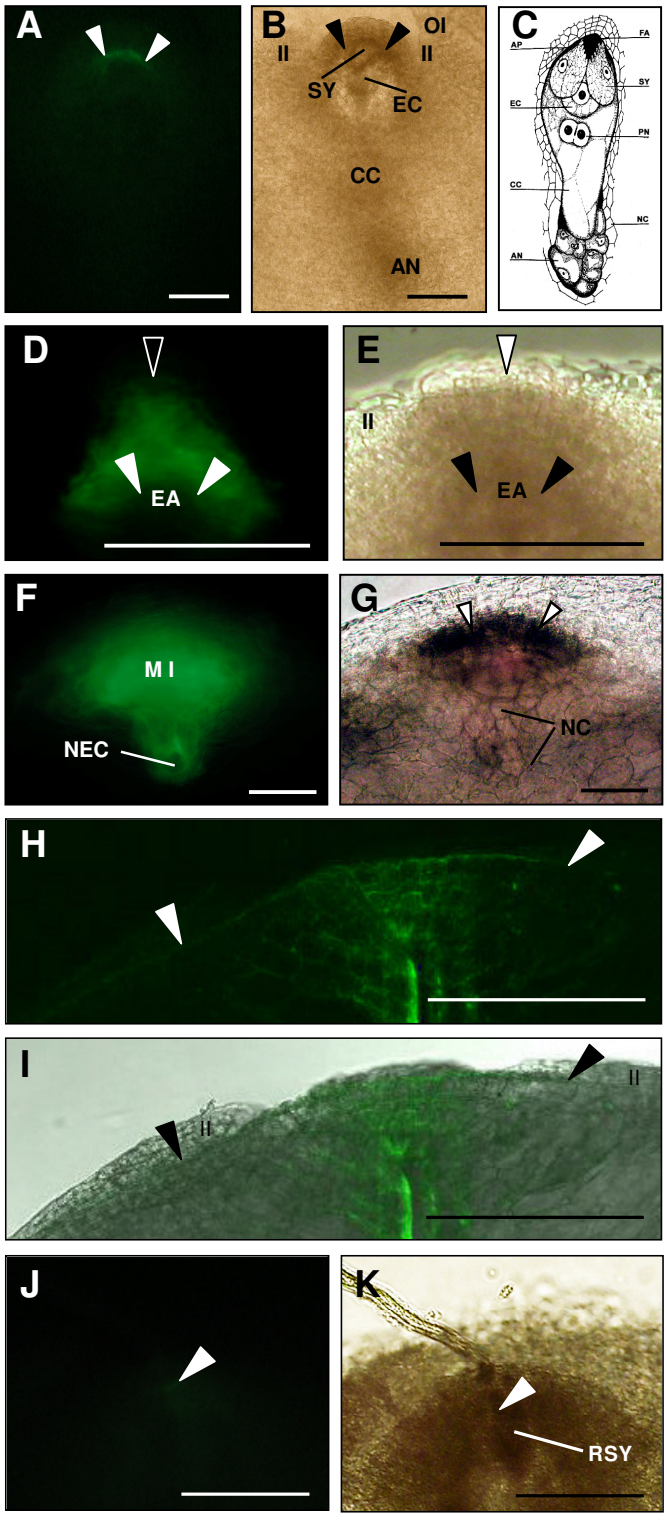

Fig. 3.17: Ovular localisation of ZmEA1:GFP fusion protein before and after *in vitro* pollination in maize.

GFP was fused to the C-terminus of ZmEA1 and transformed into maize under control of the endogenous promoter. **(A)** An unfertilised young ovule (silks <5 cm) displayed fluorescence in the synergids cell wall (arrow heads) and filiform apparatus with faint signals within the overlaying nucellus cells below the micropyle. **(B)** Light microscopic image of (B). **(C**) Schematic representation of the female gametophyte (FG) from maize, after Diboll and Larson (1966). **(D)** A fluorescence gradient was observed at the

micropylar region of unfertilised mature ovules (silks >10cm) between cell wall material of the synergids (closed arrow heads) and the surface of the nucellus cells below the micropyle (open arrow heads). **(E)** Light microscopic image of (D). **(F)** View of (D) from the opposite side through the nucellar tissue focussing on the fusion protein within the ER of the egg apparatus. Fusion protein within the micropyle is out of focus. **(G)** Light microscopic image of (F). **(H)** Confocal laser scanning microscopy (CLSM) image of an unfertilised mature ovule. Fluorescence is visible within the cell walls of the six nucellus cell layers of the micropyle. Fusion protein accumulated at the surface of the micropyle, but not at the surface of the inner integuments, as shown by the combination of CLSM and light microscopic image **(I)**. Arrow heads in (H) and (I) mark the surface of the nucellus cells underlying the inner integument. **(J)** 24 h after *in vitro* pollination, fluorescence has been mostly disappeared. A faint signal was still visible in the filiform apparatus (arrow head) and in the degenerated synergid. **(K)** Light microscopic image of (J), where a pollen tube penetrated the micropyle and released its content into the receptive and degenerated synergid. (AN: antipodals, AP: apical pocket, EA: egg apparatus, EC: egg cell, CC: central cell, FA: filiform apparatus, II: inner integument, NEC: nucleus of egg cell, NC: nucellus cells, MI: micropyle, OI: outer integument, PN: polar nuclei, RSY: receptive synergid, SY: synergids. Bars: 100 µm).

3.6.3 Subcellular localisation of ZmEA1:GFP fusion protein in onion epidermal cells

To study the subcellular localisation of the predicted ZmEA1 peptide, onion epidermal cells were used as target cells for a transient transformation assay. Transient transformation assays were performed with the pLG-ZmEA1 construct (*Ubi*p::*ZmEA1-GFP*::*NOS*t*)* that was delivered into onion epidermal cells by particle bombardment. ZmEA1:GFP fusion protein expression under the control of the constitutive ubiquitin promoter of maize was monitored 16-24 h after bombardment. All fluorescent cells investigated displayed very similar GFP fluorescence pattern. ZmEA1:GFP fusion protein was localised in the endoplasmic reticulum surrounding the nucleus, in transvacuolar strands as well as in vesicles within the cytoplasm (Fig. 3.18 A). Large quantities of the fusion protein accumulated at the cell surface, mostly associated with large vesicles (Fig. 3.18 B). The fusion protein could not be clearly detected in the cell wall. As a control for GFP expression, the construct pLNU-GFP (*Ubi*p::*GFP*) was transiently transformed into onion epidermal cells, using the same conditions. Fluorescence was visible in the nucleus and the cytoplasm (Fig. 3.18 C).

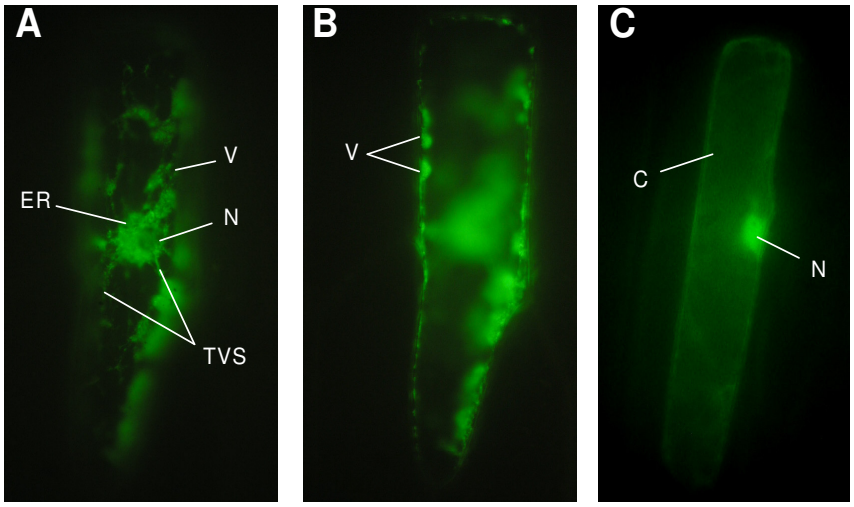

Fig. 3.18: Transient expression of a ZmEA1:GFP fusion protein in onion epidermal cells using a constitutive ubiquitin promoter of maize.

GFP expression was observed in all images 16-24 hours after bombardment. **(A)** ZmEA1:GFP fusion protein is localised at the endoplasmic reticulum surrounding the nucleus, in transvacuolar strands as well as within vesicles. **(B)** ZmEA1:GFP accumulates at the cell surface, partly associated with large vesicles within the cytoplasm. (B) shows a different focus of (A). **(C)** shows a control for GFP expression (*Ubi*p::*GFP*). GFP alone is visible in cytoplasm and nucleus. (C: cytoplasm, ER: endoplasmic reticulum, N: nucleus, TVS: transvacuolar strands, V: vacuoles)

3.7 Functional analysis of *ZmEA1* using RNAi and AS technology

A reverse genetics approach was used to study the function of *ZmEA1* in maize, as the gene maps to a chromosomal region lacking phenotypic markers (3.4.5) and insertions lines are not available. Thus, RNA interference (RNAi) and antisense (AS) approaches have been applied with the goal to study effects of *ZmEA1* activity in maize.

3.7.1 Generation of transgenic maize plants containing *ZmEA1*-RNAi and -AS constructs and molecular analysis

As shown in Table 3.4, eleven independent biolistic transformation experiments were conducted with 330 immature embryos from crosses between the maize inbred lines A188xH99, and 973 immature embryos of self-pollinated A188 plants. Embryos were bombarded with the RNAi-plasmid pZE-RNAi (*Ubi*p::*ZmEA1*-AS::*iF2*intron::*ZmEA1*::*OCS*t). In addition to the RNAi construct, the plasmid p35S-PAT carrying the *pat* gene for the glufosinate ammonium herbicide selection, was introduced

by co-transformation. The regenerated maize plants originating from inbred embryos (A188xA188) were named R-plants and those originating from hybrid embryos (A188xH99) were named Rh-plants.

In addition, 300 and 779 immature embryos originating from crossed (A188XH99) and selfed cobs (A188) were bombarded in 10 independent experiments with the plasmid pZE-AS (*Ubi*p::*ZmEA1*-AS::*NOS*t). The plasmid p35S-PAT was co-transformed together with the antisense construct. The regenerated maize plants originating from inbred embryos (A188xA188) were named AS-plants and those from hybrid embryos (A188xH99) were named ASh-plants.

In order to investigate the integration pattern and the number of transgene copies, regenerated herbicide resistant plants were analysed by genomic Southern blot analyses. Leaf genomic DNA was isolated and restricted with a pair of restriction endonucleases cutting the 5´-end of the ubiquitin promoter (*Not*I for RNAi-plants and *Sac*I for AS-plants) and the 3´-end of the OCS- and NOS-terminator, respectively (*Bsp*TI for RNAi-plants and *Kpn*I for AS-plants). After separation in agarose gels and capillary transfer to nylon membranes, DNA was hybridised with a *ZmEA1*-specific probe (Fig. 3.19).

Table 3.4 shows an overview of the maize transformations performed with the plasmid pZE-RNAi and the plasmid pZE-AS. In total, 17 *ZmEA1* transgenic lines from 1752 inbred embryos and 16 lines from 630 hybrid embryos were obtained. Thus, transformation efficiency with hybrid embryos (A188xH99) was 2.6 times higher compared with inbred embryos (A188xA188).

Table 3.4: Overview of maize transformation and regeneration. The plasmids pZE-RNAi and pZE-AS were co-transformed with the plasmid p35S-PAT. Twenty-four *ZmEA1* transgenic lines were obtained with the RNAi and nine with the AS constructs.

Approach (construct)	Origin of embryos	Number of experiments	Number of bombarded embryos	Number of regenerated lines	Number of transgenic lines* (independent clonal lines)
RNAi	A188xA188	7	973	14	14 (5)
(pZE-RNAi)	A188xH99	4	330	10	10 (8)
	Total	**11**	**1303**	**24**	**24 (13)**
AS	A188xA188	6	779	3	3 (1)
(pZE-AS)	A188xH99	4	300	8	6 (5)
	Total	**10**	**1079**	**11**	**9 (6)**

* *ZmEA1* transgenic lines

As shown in Table 3.4 and Fig. 3.19 A, twenty-four RNAi (R/Rh) T0 transgenic lines were generated that contained multiple (≥2 to ≥10) complete and/or incomplete transgene insertions in multiple loci (most lines). Lines R9, R10, R12, Rh3, Rh4 and Rh10 had only incomplete transgene integrations. Some of the RNAi transgenic lines were clonal (lines with identical transgene integration pattern), e.g. R13/4 and R13.8 (Fig. 3.19 A), as well as R1.1 and R1.2 (data not shown). Full-length RNAi transgene integrations (3,819 bp), corresponding to the *Not*I-*Bsp*TI fragment (Fig. 3.19 C) of plasmid pZE-RNAi, were detected in 7 independent T0 transgenic lines. A single transgene integration event was not detected. Using the *ZmEA1*-specific probe, the endogenous *ZmEA1* gene from maize inbred line A188 was also detected in all RNAi and wild type (wt) plants. As expected, the endogenous *ZmEA1* gene from the inbred line H99 was detected only in recombinant hybrid plants (Rh-plants).

With the exception of R12 and Rh10 (both plants died during the process of green house transfer), all RNAi T0- plants were grown to maturity and in most cases were self-pollinated or crossed with wt (A188) pollen and used as pollen donor to fertilise wt maize inbred line A188. Resulting T1-progenies were first analysed by genomic Southern blots for the integration and segregation of the transgene. Fig. 3.19 B shows a Southern blot performed with T1-plants of RNAi-lines R13.8 and Rh15 as an example.

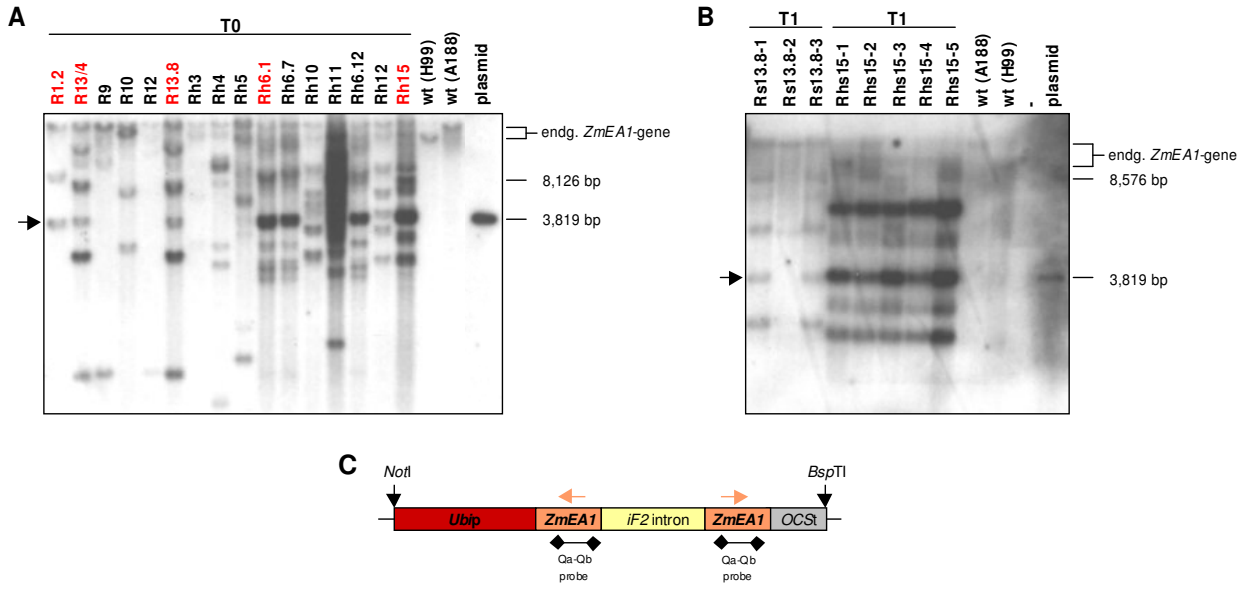

Fig. 3.19: Integration pattern of T0 and T1 maize plants transformed with the RNAi-construct *Ubi*p::*ZmEA1*-AS::*iF2*intron::*ZmEA1*::*OCS*t (pZE-RNAi).

(A) and **(B)** show genomic Southern blots performed with RNAi-plants from the T0- and T1-generation, respectively, which survived the glufosinate ammonium herbicide selection. Genomic DNA (20-25 µg)

from leaves of maize plants indicated was restricted with *Not*I and *Bsp*TI, separated in 0.8 % agarose gels, transferred onto nylon membranes and hybridised with a *ZmEA1*-specific probe. The band of 3,819 bp (arrow) represents the complete RNAi transgene integration corresponding to the *Not*I-*Bsp*TI fragment **(C)** of the plasmid pZE-RNAi. Signals corresponding to the endogenous *ZmEA1*-gene were detected in all plants analysed. 10 pg of the plasmid pZE-RNAi restricted with *Not*I and *Bsp*TI was used as a positive control. Genomic DNAs from wild type (wt) maize inbred lines A188 and H99 restricted with *Not*I and *Bsp*TI were used as negative controls. Five different transgenic lines marked in red (in A) were further analysed in the T1-generation. (endg.: endogenous).

Nine antisense (AS/ASh) T0 transgenic lines were obtained and all had multiple (≥3 to ≥8) complete (2,332 bp) and incomplete transgenes integrated in their genome (Fig. 3.20 A). Some of the AS transgenic plants were clonal (AS1.1, AS1.2 and AS1.3, ASh1.1 and ASh1.2). Full-length AS transgene integrations (2,332 bp), corresponding to the *Sac*I-*Kpn*I fragment (Fig. 3.20 B) of plasmid pZE-AS, were detected in six independent T0 transgenic lines. A single transgene integration event was not observed. The endogenous *ZmEA1* gene from maize inbred lines A188 and/or H99, was detected in almost all the maize plants transformed with pZE-AS (Fig. 3.20 A). Two plants (Ash10.1 and ASh10.2) showed only the endogenous gene, thus did not contain the transgene. All transgenic maize plants were grown to maturity and in most cases were self-pollinated and used to pollinate wt maize inbred line A188.

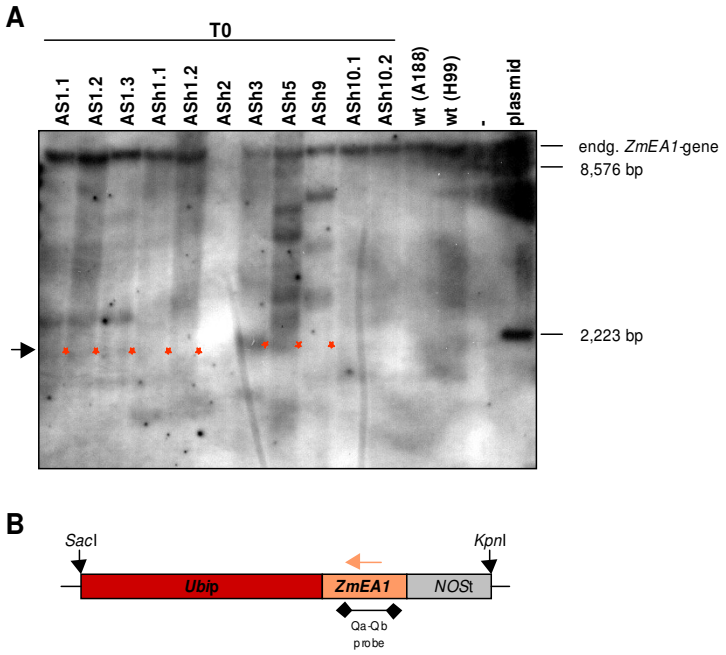

Fig. 3.20: Integration pattern of T0 maize plants transformed with the antisense construct *Ubi*p::*ZmEA1*-AS::*NOS*t (pZE-AS). (legend continued on the next page)

(A) shows a genomic Southern blot performed with pZE-AS-transformed maize plants from the T0-generation, which survived the glufosinate ammonium herbicide selection. Genomic DNA (20-25 µg) from leaves of plants indicated was restricted with *Sac*I and *Kpn*I and separated in a 0.8 % agarose gel. Following transfer onto a nylon membrane, digested DNAs were hybridised with a *ZmEA1*-specific probe. The band of 2,332 bp (arrow) represents the complete AS transgene integration corresponding to the fragment *Sac*I-*Kpn*I **(B)** of plasmid pZE-AS. Hybridisation to the endogenous *ZmEA1*-gene was detected in almost all plants analysed. 10 pg of the plasmid pZE-AS restricted with *Sac*I and *Kpn*I was used as a positive control. Genomic DNAs from wild type (wt) maize inbred lines A188 and H99 were restricted with *Sac*I and *Kpn*I and used as negative controls and to show the endogenous *ZmEA1* gene. (endg.: endogenous)

3.7.2 Phenotypic analysis of transgenic maize lines containing RNAi and AS constructs

All RNAi and AS T0 transgenic plants were selfed or crossed with wt (A188) pollen to monitor seed set. Additionally, most of them were also used as pollen donor to fertilise wt (A188) plants. An overview of seed set is shown in Table 3.5. Six out of twelve transgenic lines with complete transgene integrations showed a significant reduction of seed set (0-75%) upon selfing. The female sterility effect was especially strong in transgenic inbreds (seed set was reduced to 0-30%). In hybrid transgenic lines, seed set was only slightly reduced, varying from 25-95%. The four RNAi lines lacking complete transgene integrations (R9, R10, Rh3 and Rh4) displayed a seed set comparable to wt (95-100%). Clonal transgenic plants displayed very similar seed set. Seed set of the selfed or with wt pollen crossed clonal line R13/2, R13/3, R13/4, R13/5, R13/8, R13.1, R13.2, R13.6, R13.8 was between 0-10%, while seed set of another clonal line R1.1, R1.2 was reduced to 20-30%. In contrast, seed set of the clonal line Rh6.1, Rh6.7, Rh6.12 was 80-95%, almost comparable to the wt. All wt plants crossed with transgenic pollen gave also a full seed set indicating that not the male gametophyte (MG) but potentially the female gametophyte (FG) is affected by the lower *ZmEA1* activities. A small number of transgenic plants showed male sterility (R13/2, AS1.1, AS1.2 and AS1.3) and were also relatively small, probably an effect of the tissue culture procedure.

Table 3.5: Overview of Southern blots and phenotypic analyses of T0-generation maize plants containing *ZmEA1*-specific RNAi and AS constructs.

The number of transgene integrations as well as seed set in percentage of T0 cobs is indicated. Are shown 11 independent clonal lines obtained with the RNAi and six with the AS constructs. Each block represents an independent clonal line. T0 cobs were generated by self-pollination or by out-crossing with pollen from the inbred line A188 and/or by using the pollen to fertilise A188 cobs. Transgenic lines lacking full integrations are underlined in grey. (ms: male sterility: either there were no anthers, pollen missing or the pollen was not vital; pn: pollen morphologically normal).

A: T0 generation originating from inbred embryos (A188 x A188)

Approach	Plant line	Number of transgene integrations	Full-length transgene integration	Seed set in T0 cobs (%)		
				Ovules		Pollen (crossed to A188)
				selfed	out-crossed	
RNAi (R)	R1.1	≥ 2	yes	30%	-	100%
	R1.2	≥ 2	yes	20%	-	100%
	R13/2	≥ 6	yes	-	0%	ms
	R13/3	≥ 6	yes	-	0%	pn
	R13/4	≥ 6	yes	0%	-	100%
	R13/5	≥ 6	yes	0%	-	100%
	R13/8	≥ 6	yes	0%	-	pn
	R13.1	≥ 6	yes	-	0%	pn
	R13.2	≥ 6	yes	10%	-	pn
	R13.6	≥ 6	yes	-	10%	pn
	R13.8	≥ 6	yes	5%	-	pn
	R9	≥ 3	no	100%	-	100%
	R10	≥ 3	no	95%	-	100%
AS	AS1.1	≥ 7	yes	-	0%	ms
	AS1.2	≥ 7	yes	-	0%	ms
	AS1.3	≥ 7	yes	-	0%	ms

B: T0 generation originating from hybrid embryos (A188 x H99)

Approach	Plant line	Number of transgene integrations	Full-length transgene integration	Seed set in T0 cobs (%)		
				Ovules		Pollen (crossed to A188)
				selfed	out-crossed	
RNAi (R)	Rh5	≥ 7	yes	85%	-	pn
	Rh6.1	≥ 9	yes	95%	-	pn
	Rh6.7	≥ 9	yes	95%	-	pn
	Rh6.12	≥ 9	yes	80%	-	pn
	Rh11	≥ 10	yes	100%	-	pn
	Rh12	≥ 5	yes	-	75%	pn
	Rh15	≥ 8	yes	90%	-	pn
	Rh3	≥ 4	no	100%	-	100%
	Rh4	≥ 6	no	100%	-	pn
AS	ASh1.1 – 1st cob	≥ 8	yes	0%	-	20%
	ASh1.1 – 2nd cob		yes	-	10%	
	ASh1.2	≥ 8	yes	75%	-	100%
	ASh2	≥ 5	yes	70%	-	100%
	ASh3	≥ 3	yes	100%	-	100%
	ASh5	≥ 7	yes	65%	-	90%
	ASh9	≥ 6	yes	25%	-	100%

Figure 3.21 shows examples of T1 segregation for female sterility of some RNAi lines (R1.1, R1.2, R13/4 and R13/5; Fig. 3.21 A and B) and AS lines (Ash5 and Ash9; Fig. 3.21 C and D) containing full-length transgene integrations. It is obviously that seed set is significantly reduced after selfing, compared with 100% seed set obtained after using

pollen of RNAi and AS-lines to fertilise A188 wt plants, indicating that the FG, but not the MG is affected. In some cases the female sterility effect was very strong and selfed-cobs did not contain any seed (Rs13/4 and Rs13/5 cobs in Fig. 3.21 B).

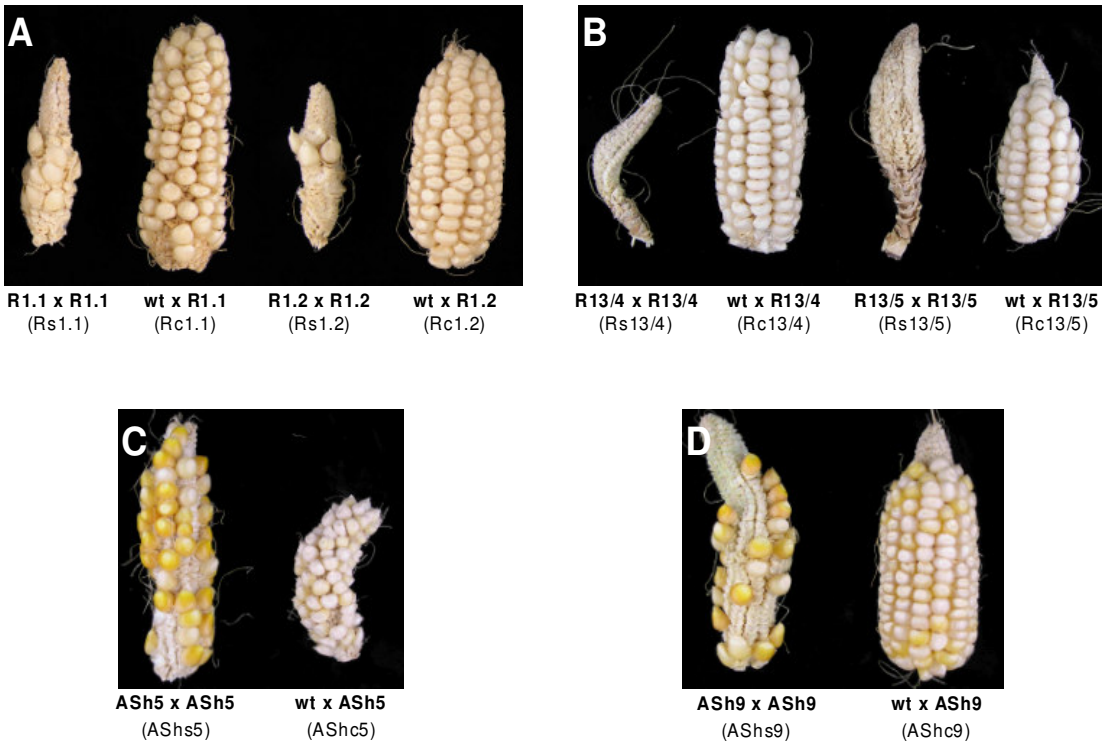

Fig. 3.21: T1 segregation for female sterility of RNAi and AS lines.
(A) and **(B)** show T1 segregation of clonal RNAi lines R1.1 and R1.2, and of clonal RNAi lines R13/4 and R13/5, respectively, containing full-length transgene integrations. In both images, the first two cobs starting from the very left belong to one clonal line and the following two cobs belong to the other clonal line. For both lines, always the first cob from the left has been self-pollinated and the second cob generated after using the pollen of the RNAi line indicated to fertilise a wild type (wt) cob (A188). **(C)** and **(D)** show T1 segregation of two different antisense transgenic maize lines ASh5 and ASh9 containing complete transgene integrations. The cob at very left has been self-pollinated and the right cob represents a wt (A188) cob pollinated with pollen from the AS line indicated.

The RNAi-lines R1.1, R1.2, R13.8, R13/4, Rh6.1 and Rh15, all containing complete transgene integrations, were further analysed in the T1-generation. After herbicide selection, T1 plants of these lines were analysed by Southern blots for the integration of the transgene (Fig. 3.19 B shows an example) and then phenotypic analysed. An overview of the results is shown in Table 3.6. In general, a correlation between glufosinate ammonium resistance and integration of the transgene was found for almost all transgenic lines tested. Most of the analysed T1 plants with complete transgene

integrations showed reduced seed set after selfing or after out-crossing with wt (A188) pollen (0-65%), but showed full seed set after using them as pollen donor to fertilise wt plants. Unless mentioned, pollen of the transgenic and wt plants were morphologically normal. This female sterility effect observed in the T2 segregation of RNAi lines further supports the hypothesis that the female gametophyte, but not the male gametophyte is affected, when the *ZmEA1* activity is potentially reduced. Transgenic T1-plants originating from selfed-T0 RNAi lines R1.1 and R1.2 as well as some originating from crossed-T0 line R1.2 showed a different phenotype of female sterility (no cob or no silk) and/or male sterility (no anthers or no pollen or the pollen was not vital) or a dwarf phenotype (plants Rs1.2-1, Rs1.2-2, Rs1.2-3, Rs1.2-4, data not shown). These pleiotropic phenotypes are probably originating from the tissue culture procedure and/or abiotic factors rather then from the transgene. In general, the transgene was segregating within the T1 progenies originating from the same T0-line, indicating that transgene integrated into different loci.

Table 3.6: Overview of Southern blot and phenotypic analyses of T1-generation maize plants containing the *ZmEA1*-specific RNAi construct.

The number of transgene integrations as well as the seed set in percentage of T1-cobs is indicated. Are grouped in one block T1-plants generated from the same T0-line. T1 cobs were generated by self-pollination as well as out-crossing with A188 or H99 maize lines and by using the pollen to fertilise A188 plants. Marked in red are T1-plants used for *in vitro* pollination assays and/or Q RT-PCR analyses (ms: male sterility: either there were no anthers, pollen missing or the pollen was not vital; pn: pollen morphologically normal)

A: T1 generation from selfed T0-lines

T0- donors	T1- plant	Number of transgene integrations	Full-length transgene integration	Seed set in T1 cobs (%)		
				Ovules		Pollen (crossed to A188)
				selfed	outcrossed	
Rs1.1 (R1.1 x R1.1)	Rs1.1-1	≥ 3	Yes	no cob	-	ms
	Rs1.1-2	≥ 3	Yes	no silk	-	ms
	Rs1.1-3	≥ 1	No	no cob	-	ms
	Rs1.1-4	≥ 3	Yes	no cob	-	ms
	Rs1.1-5	≥ 3	Yes	100%	-	100%
	Rs1.1-6	≥ 3	Yes	no cob	-	ms
Rs1.2 (R1.2 x R1.2)	Rs1.2-1	≥ 4	Yes	no cob	-	pn
	Rs1.2-2	≥ 4	Yes	no cob	-	ms
	Rs1.2-3	≥ 3	Yes	no cob	-	pn
	Rs1.2-4	≥ 4	Yes	no cob	-	pn
	Rs1.2-5	≥ 5	Yes	no cob	-	ms
	Rs1.2-6	≥ 4	Yes	no silk	-	pn
	Rs1.2-7	≥ 4	Yes	no cob	-	0%
	Rs1.2-8	≥ 4	Yes	no silk	-	95%
	Rs1.2-9	≥ 4	Yes	no silk	-	100%
	Rs1.2-10	≥ 4	Yes	no silk	-	10%
	Rs1.2-13	≥ 2	Yes	no silk	-	ms
Rs13.8 (R13.8 x R13.8)	Rs13.8-1	≥ 6	Yes	0%	-	100%
	Rs13.8.3	≥ 6	Yes	0%	-	100%

Table 3.6 A (continued)

T0- donors	T1- plant	Number of transgene integrations	Full-length transgene integration	Seed set in T1 cobs (%)		
				Ovules		Pollen (crossed to A188)
Rhs6.1 (Rh6.1 x Rh6.1)	Rhs6.1-1	≥ 8	Yes	*in vitro* pollination assay	-	pn
	Rhs6.1-2	≥ 8	Yes	-	0%	pn
	Rhs6.1-3	≥ 8	Yes	-	40%	pn
	Rhs6.1-4	≥ 8	Yes	12%	-	100%
	Rhs6.1-5	≥ 8	Yes	95%	-	pn
	Rhs6.1-6	≥ 9	Yes	0%	-	80%
	Rhs6.1-7	≥ 9	Yes	100%	-	pn
	Rhs6.1-8	≥ 9	Yes	-	85%	pn
	Rhs6.1-9	≥ 9	Yes	95%	-	100%
Rhs15 (Rh15 x Rh15)	Rhs15-1	≥ 6	Yes	100%	-	100%
	Rhs15-2	≥ 6	Yes	0%	-	pn
	Rhs15-3 – 1st cob	≥ 8	Yes	100%	-	100%
	Rhs15-3 – 2nd cob			*in vitro* pollination assay		
	Rhs15-4	≥ 5	Yes	*in vitro* pollination assay	-	pn
	Rhs15-6	≥ 2	No	80%	-	100%
	Rhs15-7	≥ 5	Yes	65%	-	pn
	Rhs15-8	≥ 5	Yes	-	0%	pn
	Rhs15-9	≥ 5	Yes	-	100%	pn
	Rhs15-10	≥ 5	Yes	no cob	-	pn
	Rhs15-11	≥ 5	Yes	-	95%	pn
	Rhs15-12	≥ 5	Yes	Q RT- PCR analysis + *in vitro* pollination assay	-	pn
	Rhs15-13	≥ 5	Yes	-	0%	pn

B: T1 generation from crossed T0-lines

T0- donors	T1- plant	Number of transgene integrations	Full-length transgene integration	Seed set in T1 cobs (%)		
				Ovules		Pollen (crossed to A188)
				selfed	outcrossed	
Rc1.1 (A188 x R1.1)	Rc1.1-1	≥ 1	No	100%	-	100%
	Rc1.1-2	≥ 1	No	-	80%	pn
	Rc1.1-3	≥ 3	Yes	-	50%	100%
	Rc1.1-4	≥ 1	No	-	95%	pn
	Rc1.1-5	≥ 1	No	-	90%	pn
	Rc1.1-6	≥ 1	No	-	50%	pn
Rc1.2 (A188 x R1.2)	Rc1.2-1	≥ 5	Yes	no silk	-	pn
	Rc1.2-2	≥ 5	Yes	no cob	-	pn
	Rc1.2-3	≥ 5	Yes	no silk	-	pn
	Rc1.2-4	≥ 2	Yes	100%	-	50%
	Rc1.2-7	≥ 2	Yes	100%	-	100%
	Rc1.2-9	≥ 2	Yes	-	0%	75%
Rc13/4 (A188 x R13/4)	Rc13/4-1	≥ 2	?	100%	-	pn
	Rc13/4-2	≥ 2	?	100%	-	pn
	Rc13/4-3	≥ 2	?	3%	-	pn
	Rc13/4-4	≥ 2	?	100%	-	pn
	Rc13/4-5	≥ 2	?	65%	-	pn

Examples of T1-cobs segregating for female sterility (T2-segregation) are shown in Fig. 3.22. For transgenic line R1.1, it could be noticed that the seed set is significantly reduced after out-crossing with wt (A188) pollen, but full seed set (100%) after using as pollen donor to fertilise wt (A188) plants) (Fig. 3.22 B). This female sterility phenotype observed in the T2-segregation is similar with the one of the T1-segregation (Fig. 3.21 A; Fig. 3.22 A), indicating again that the female gametophyte, but not the male gametophyte is affected by the lower *ZmEA1* activities. In case of transgenic line R13.8, the T1 selfed-cobs did not contain any kernels, while the T1-crossed cobs had full seed set (Fig. 3.22 D). The corresponding T0 selfed-cob contained few seeds (Fig. 3.22 C).

An explanation for this strong female sterility effect observed in the T2-segregation of line R13.8 could be that the plants analysed were homozygous. In addition, segregation of the transgene was not observed within the T1-progenies of this line (Table 3.6) indicating that the transgene might be integrated at a single-locus.

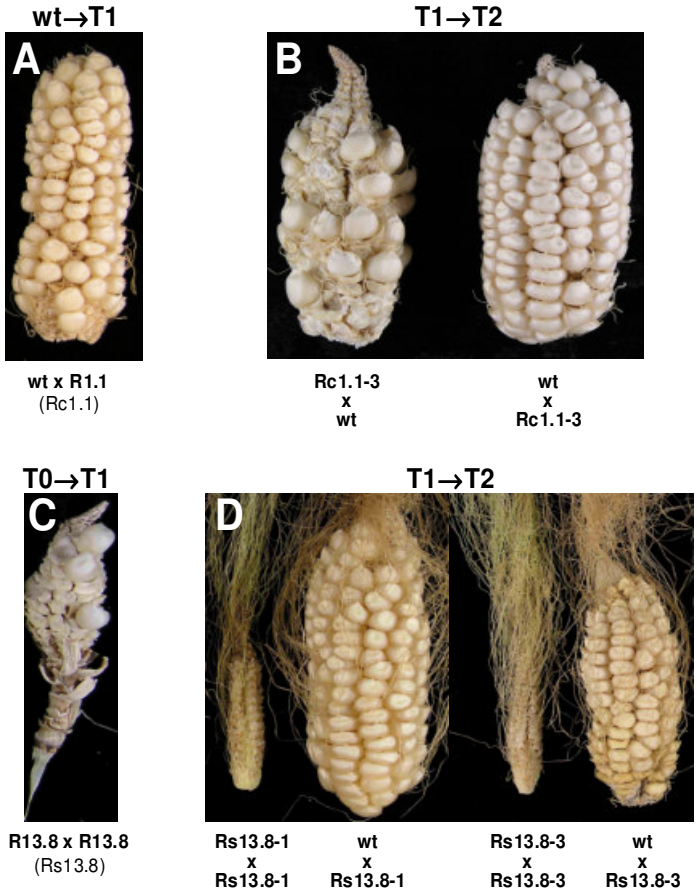

Fig. 3.22: T2 segregation for female sterility of RNAi lines.

(A) shows a wild type (wt) cob (A188) pollinated with pollen of the RNAi line R1.1 containing complete transgene integrations. In **(B)** is shown T2 segregation of one transgenic progeny plant (Rc1.1-3) originating from (A). The cob at the very left was crossed with wt (A188) pollen and the right cob represents a wt (A188) cob pollinated with pollen from the RNAi line indicated. **(C)** shows the T0 selfed-cob of the RNAi line R13.8 containing complete transgene integrations. **(D)** shows T2-segregation of two transgenic progenies plants originating from (C), where the first two cobs starting from the very left belong to one progeny and the following two cobs belong to the other one. For both progenies, always the first cob from the left was self-pollinated and the second cob generated after using the pollen of the RNAi line indicated to fertilise a wt cob (A188).

3.7.3 Morphology of female gametophyte of RNAi lines and *ZmEA1* expression levels in transgenic egg cells

With the aim to study the cause of female sterility, ovule sections and embryo sacs were microdissected from RNAi T1 and wt (A188) cobs and analysed by light microscopy. No morphological differences could be observed between the female reproductive structures of RNAi and wt lines (Fig. 3.23 A and B).

To quantify *ZmEA1* gene-silencing effect, single cell Q RT-PCR analysis was performed with isolated egg cells from the Rhs15-12 T1 (Table 3.6 A) and wt (A188) cobs using endogenous *ZmEA1*-specific primers. The plant Rhs15-12 contains complete RNAi transgene integrations and it showed less then 50% fertilisation efficiency after *in vitro* pollination assays (Fig. 3.24 E). This plant has not been proven to be homozygous for the transgene. *GAPDH*-specific primers or primers specific to the female gametophyte marker gene *ZmES1* (Amien, 2003) were used as internal controls. Egg cells without the RT step as well as water controls were used to control amplification from genomic DNA or contaminations. PCRs results were controlled by agarose gel-electrophoresis. Only data from samples showing both *ZmEA1*- and *GAPDH*- or *ZmEA1*- and *ZmES1*-specific amplifications were processed. *GAPDH*- and *ZmES1*-specific PCR products were used to normalise *ZmEA1* transcript amounts. As shown in Fig. 3.23 C, significant down regulation of the *ZmEA1* gene was observed in five of nine egg cells isolated from the RNAi-plant Rhs15-12. The egg cells number 7, 8 and 9 showed a *ZmEA1* gene-silencing effect of about 42%, 55% and 61%, respectively, relative to the wt expression level. For egg cells number 5 and 6 the silencing effect was only about 14% and 17%, respectively. The RNAi egg cells number 1 to 4 did not show a down regulated *ZmEA1* gene. Interestingly, the values obtained for egg cells number 1, 2 and 3 were higher compared to the wild type control. The corresponding T0-line of the RNAi-plant Rhs 15-12 was generated from hybrids embryos of a cross between the inbred lines A188 and H99 (Table 3.5 B). It is thus possible that *ZmEA1* transcript levels are higher in egg cells of hybrid plants compared to egg cells from inbred lines such as A188, which should be investigated during further experimentation.

In summary, a significant down regulation of the *ZmEA1* gene was determined, although none of the egg cells displayed a full knock-out.

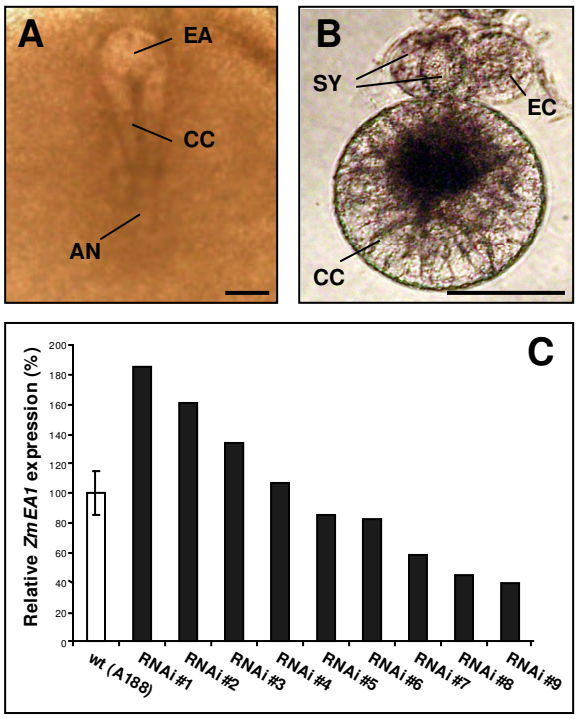

Fig. 3.23: Morphology of female gametophyte of RNAi lines and quantification of *ZmEA1* transcript amounts from egg cells isolated from a maize plant containing full-length transgene integrations and from wt egg cells.

(A) Section through an ovule of an RNAi line with reduced fertility (Rhs15-4, Fig. 3.24 E) showing a wild type (wt)-like embryo sac. **(B)** The embryo sac from (A) after microdissection showed developed fully differentiated and mature cells. **(C)** Relative *ZmEA1* expression in wt and RNAi plants. Single cell Q RT-PCR analysis was performed with egg cells from the RNAi-plant Rhs15-12 and with wt (A188) egg cells using *ZmEA1*- and *GAPDH*- or *ZmEA1*- and *ZmES1*-specific primers. *GAPDH*- and *ZmES1*-specific PCR products were used to normalise *ZmEA1* transcript amounts. Eight egg cells of the inbred line A188 were used to determine the relative expression of *ZmEA1* in wt plants. The *ZmEA1* gene was significantly down regulated in egg cells number 7, 8 and 9 and weaker in egg cells number 5 and 6, from the RNAi line. A silencing effect could not be observed for egg cells number 1 to 4, instead, expression is partly even higher than in wt egg cells. (AN: antipodals, EA: egg apparatus, EC: egg cell, CC: central cell, SY: synergids. Bars: 100 µm).

3.7.4 *In vitro* pollination assays and fertilisation efficiencies with maize lines containing the RNAi construct

Morphologically, the female reproductive structures of RNAi lines were not distinct from that of wt plants (3.7.3). Thus, to visualise pollen tube (PT) growth and investigate the fertilisation process in order to identify the cause of female sterility, *in vitro* pollination assays were made with progenies originating from T0-selfed cobs of two RNAi lines (Rh6.1 and Rh15 containing complete transgene integrations and showing only a slight

reduction of seed set, Table 3.5 B) and with A188 maize cobs as controls. Pollen were used either from a homozygous maize line expressing *GUS* under the control of the rice actin promoter (Brettschneider *et al.,* 1997) to fertilise plants Rhs6.1-1, Rhs15-3, Rhs15-4, or from wt (A188) plants to fertilise plant Rhs15-12 (showing significant down regulation of the *ZmEA1* gene in transgenic egg cells, Fig. 3.23 C). Aniline blue staining, which is used as a standard method to monitor PT growth in *Arabidopsis* and other species did not work sufficiently in maize, as the FG is more deeply embedded in maternal tissue and pollen tubes contain less callose. Applying the GUS staining procedure, the PT was stained blue, in most cases, and the growth could be observed within the maize ovary. 24-30 h after pollination and 24-40 h after staining, when *Actinp*::*GUS* pollen was used, the number of fertilised embryo sacs was determined by counting ovules containing a degenerating synergid and/or a pollen tube tip penetrating the micropylar region. Following pollination of wt lines, many PTs reached the surface of the inner integument and continued growth towards the micropylar region (Fig. 3.24 A), but only one PT turned abruptly at the micropyle of each ovule towards the egg apparatus where its content was released in the receptive synergid (Fig. 3.24 B). In contrast, 24-30 h after pollination of two selfed RNAi lines (Rh6.1 and Rh15), the majority of pollen tubes did not enter the micropyle although pollen tubes were visible at the surface of all ovules analysed. Fig. 3.24 D shows an example of a PT growing close to but without entering the micropyle (within a distance of 50-100 µm towards the egg apparatus). In most cases pollen tubes grew at random directions at the surface of the inner integument, as shown in Fig. 3.24 C, where the PT, but not the micropyle is in focus. These types of loss of PT guidance were never observed in wt ovules.

In summary, GUS staining was visible in 82% of wt FGs 24-30 h after *in vitro* pollination (Fig.3.24 E), but only 40-55% of the analysed ovules of RNAi lines showed blue staining within the FG. Based on the observed phenotypes, the reduction of *ZmEA1* activity in the egg apparatus seems to affect micropylar, but not long-range PT guidance in about half of the ovules.

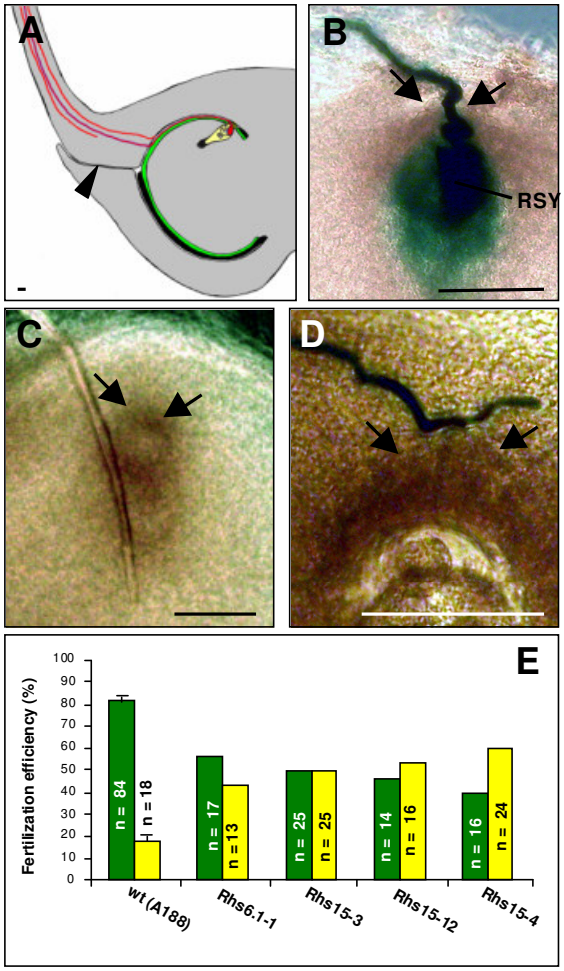

Fig. 3.24: Loss of close-range PT guidance in maize transgenic lines with reduced *ZmEA1* activity. **(A)** Scheme of pollen tube growth in maize. Each ovary encloses a single bitegmic ovule. In maize, the outer integument (black) is reduced compared to the inner integument (green) which itself encloses most of the nucellus except in the vicinity of the female gametophyte (FG, yellow). This region corresponds to the micropyle. Multiple pollen tubes (red) grow through the transmitting tissues of the silk to the inner integument, but only one pollen tube penetrates the micropyle and releases sperm cells in the receptive synergid. Arrow head points towards the stylar channel. **(B-E)** Transgenic *Actinp*::*GUS* maize pollen or wild type (wt) pollen (A188) was used to monitor pollen tube growth, sperm discharge and fertilisation in wt and transgenic lines (see text). 24-30 h after *in vitro* pollination ovules were excised, and/or stained and GUS expression was analysed in the embryo sac. **(B)** An example of a GUS expressing pollen tube growing towards the micropyle of a wt ovule. Growth direction is abruptly changed at the surface of the micropyle where the tube penetrates the cell walls of nucellus cells and releases its content into the receptive synergid. **(C** and **D)** Pollen tubes within around 50% ovaries of transgenic lines (examples show lines Rhs6.1-1 and Rhs15-3; Fig. 3.24 E) grew at the surface of the inner integument without entering the micropyle, instead continuing growth in random directions. This phenotype was never observed in wt ovules. **(E)** Summary of FG fertilisation in wt and transgenic ovules. In wild type, 82% of the embryo sacs were fertilised 24-30 h after *in vitro* pollination. GUS staining and thus fertilisation of embryo sacs from RNAi lines investigated was reduced to 40-55%. n gives the number of excised ovules showing the indicated phenotype, green colour: fertilised FGs and yellow colour: unfertilised FGs. (RSY: receptive synergid. Arrows mark the micropylar opening of the ovule. Bars: 100 µm).

4 Discussion

4.1 Female gametophyte-specific genes

Molecular and functional analysis of the female gametophyte (FG, embryo sac) is a fundamental issue in plant biology, as its development and function represent a central aspect of the plant life cycle and controls essentially every step of the reproductive process. During the fertilisation process, the FG also participates in directing the pollen tube to the ovule (Hülskamp *et al.*, 1995; Ray *et al.*, 1997; Shimizu and Okada, 2000; Higashiyama *et al.*, 2001) and mediates fertilisation of the egg and central cell (Russell, 1992).

Although extensively studied at the cytological level, little is known about the molecular and genetic processes controlling female gametophyte development and function. Some progress has been made over the past years by genetic approaches, especially in *Arabidopsis*. A number of female-gametophyte mutants have been identified, and we are beginning to understand the molecular processes regulating female gametophyte function.

This work represents an alternative approach to identify genes that play important roles in the functional processes of the female gametophyte such as pollen tube guidance and fertilisation. A reverse genetic approach was performed involving microdissection of the cells of the maize FG, generation of an egg cell-specific cDNA library (Dresselhaus *et al.*, 1994) and bioinformatical analyses of generated ESTs. The first results of the BLASTX analysis showed that among the most abundant ESTs, only one contig encodes a histone, while some contigs showed homologies to genes encoding proteins involved in translation, protein stabilisation and degradation. This confirms the finding that the unfertilised egg cell of maize is in a relatively quiescent state of low metabolic activity, but may be prepared for rapid increase in translational activity after fertilisation (Diboll, 1968; Dresselhaus *et al.*, 1999b). From 988 random clones originating from the egg cell cDNA library, 32 clones represented *ZmEA1* (*Zea mays Egg Apparatus1*) gene suggesting a high level of expression in mature egg cells. Among the most abundant contigs of this cDNA library, the genes *ZmTLA1* (*Zea mays Transparent Leaf Arrea1*) and *ZmES1-4* (*Zea mays Embryo Sac1-4*), previously characterised as gametophyte- and female gametophyte-specific genes in maize, respectively, that play a role in cell differentiation and the fertilisation process, were also identified (Dresselhaus *et al.*, unpublished; Amien, 2003).

The *ZmEA1* gene was selected for this study because it represents the second most abundant contig and did not show any homology to known genes or ESTs in public databases. *ZmEA1* did show a 100% homology to an unpublished cDNA clone from maize with a putative egg apparatus expression pattern *Zmec 135* (*Zea mays egg cell 135*). *Zmec 135* was previously shown to be down regulated after fertilisation suggesting a fertilisation related function (Cordts, 2000). More sensitive expression analysis in different maize tissues by RT-PCR confirmed this expression pattern and thus *ZmEA1* gene represents to my knowledge the only gene in plants that shows expression exclusively in the egg apparatus. All genes characterised up to date showed variable expression profiles among the cells of the female gametophyte. While *ZmES1-4* genes are expressed in all cells of the maize embryo sac and are down regulated after fertilisation (Cordts, 2001), *MEA* (*Medea*) gene is expressed in the egg cell and in the central cell, of the *Arabidopsis* female gametophyte and expression after fertilisation was detectable until the heart and torpedo stage of embryo development (Vielle-Calzada *et al.*, 1999; Luo *et al.*, 2000). The *Arabidopsis FIS2* (*Fertilisation-Independent Seed2*, Luo *et al.*, 2000) and maize *ZmEBE-1/2* (*Zea mays Embryo sac/Basal endosperm transfer layer/Embryo surrounding region-1/2*, Magnard *et al.*, 2003) genes are expressed in the FG before fertilisation only in the central cell and after fertilisation selectively expressed in the endosperm and not in the embryo. *ZmTLA1* gene was shown to be expressed in the egg cell and synergids, but also in meristematic tissues and in microspores (Dresselhaus *et al.*, unpublished), and was down regulated after fertilisation. The only similarity *ZmEA1* shares with *ZmES1-4* and *ZmTLA1* is that the genes are intronless genes and encode small peptides. It has been speculated that the lack or small introns might be viewed as advantageous for genes that are transcribed in a constitutive or in an extensive way (Carels and Bernardi, 2000). The *ZmEA1* gene is very specifically expressed, although highly expressed. In summary, *ZmEA1* represents the first gene exclusively expressed in the egg apparatus, which is down regulated after fertilisation suggesting an important function for the fertilisation process.

4.2 *ZmEA1* promoter activity in cereals

1,463 bp DNA 5' upstream of the *ZmEA1* transcription start point (TSP) was isolated as the *ZmEA1* promoter from the maize inbred line A188. Sequence analysis with different public databases identified frequent *cis*-elements involved in binding of basal

transcription factors as well as specific transcription factors indicating that the isolated fragment is a functional promoter. General transcription factors are very important for the activation of RNA polymerase II (RNA pol II), but do not have an influence on tissue-specific expression. The most basic *cis*-element is the TATA box, which is found in most eukaryotic genes located around position -30 relative to the TSP and which is responsible for positioning RNA pol II correctly to initiate transcription (Buchanan *et al.*, 2000). Another basic *cis*-element is the so called CAAT box, which influences the initiation rates of transcription and which is involved in directing the RNA pol II to the closest vicinity of the transcription start point to establish the first contacts for building the transcription initiation complex (Buchanan *et al.*, 2000). The classical TATA box (TATA(T/A)AT, Joshi, 1987) could not be identified some 32±7 nucleotides upstream of the *ZmEA1* TSP. However, a TATA-like box (TACAAAT, -31 to -25 bp) and two CAAT boxes (-83 to -80 and -92 to –89 bp) were identified 5' upstream of the TSP that could participate in the general transcription complex to promote the *ZmEA1* transcription. Interestingly, a TACAAAT box (-34 to -28 bp) and a CAAT box (-79 to -76 bp) were also identified in the *ZmTLA1* promoter, which drives gene expression in the egg apparatus (Dresselhaus *et al.*, unpublished). The presence of a direct repeated sequence was identified in the *ZmEA1* promoter and could potentially play a role in the transcriptional activation of the gene, but this has to be verified.

Numerous additional putative transcriptional regulatory elements were identified in the *ZmEA1* promoter region between positions -271 and -751 relative to the TSP. Among those elements was identified an MSA-like element (-576 to -568 bp). The MSA-element (M-specific activator) is a common *cis*-acting element found in plant B-type cyclin gene promoters and which is necessary and sufficient for the phase-specific promoter activation (Ito *et al.*, 1998; Ito, 2000). MSA-like sequences were also found in the promoters of G2/M-specific genes encoding kinesin-like proteins indicating that the MSA-mediated mechanism might be plant-specific (Ito, 2000). Interestingly, an MSA-like element was also identified in the promoters of the genes *ZmES1* and *ZmES4* (Amien, 2003) as well as *ZmTLA1* (Dresselhaus *et al.*, unpublished). No additional significant homology was identified between the promoters of *ZmEA1*, *ZmES1/4* and *ZmTLA1*, although all of them are expressed in the female gametophyte of maize and belong to the most abundant contigs of the egg cell cDNA library. The MSA-like regulatory element identified in the promoters of these four female gametophyte-specific genes could be probably involved in regulation of the cell cycle progression in the maize egg

cell before fertilisation. Egg cells are resting cells prepared to initiate development into an embryo once they become activated by a sperm cell (Dresselhaus *et al.*, 1999b). For fertilisation to be successful, the egg cells need to be in a specific phase of the cell cycle before fusion, which in tobacco (*Nicotiana tabaccum*) it was shown to be G2, and in maize and other members of the grass family tend to be G1 (Weterings and Russell, 2004). Other elements identified in the *ZmEA1* promoter include a Myb26 binding site located 141 nucleotides further upstream of the MSA-like element. Myb26 is a MYB-like protein identified in pea flowers with affinity for promoters of phenylpropanoid biosynthetic genes (Uimari and Strommer, 1997). A set of data in the literature suggest the possibility that plant Myb may have important roles in G2/M by inducing B-type cyclin genes, together with other cell cycle-related genes in plants (Ito, 2000). *ZmEA1* and the other three female gametophyte-specific genes described above are all down regulated after fertilisation. Probably, the transcription level of these genes before and after fertilisation may be regulated by plant transcriptional factors, which are related to the animal Myb oncoproteins.

Defined promoter fragments were fused to the *uidA* reporter gene to demonstrate transcriptional activity of the *ZmEA1* 5' flanking region. *GUS* marker gene expression under the control of the longest *ZmEA1* promoter fragment was exclusively detected in the egg apparatus of maize demonstrating the functionality of the *ZmEA1* promoter. This also confirms the *ZmEA1* expression pattern in the maize egg apparatus previously obtained by *in situ* hybridisation using semi-thin ovule sections of maize and single cell whole mount *in situ* hybridisation with isolated cells of the FG (Cordts, 2000). In rice, histochemical analyses performed with ovules of transgenic lines did not show detectable *GUS* activities. However, after performing RT-PCR analyses with different transgenic rice lines containing the *ZmEA1* full-length promoter, *GUS* transcripts were detected in all lines. This demonstrates that the longest *ZmEA1* promoter fragment is functional also in rice. The failure to detect *GUS* expression in rice female gametophytes might be due to technical reasons, as the GUS staining solution used might not be able to penetrate the embryo sac of rice. This could be improved by adding more detergent to the GUS staining solution and/or decreasing its osmolarity. The maize line harbouring the *ZmEA1* full-length promoter-*GUS* fusion could now be used as a marker line in the analysis of maize female gametophytes mutants, especially if they generate aberrations in the egg apparatus, e.g. lack of cell development, maturation or identity. The *ZmEA1* promoter may also be used to express different

target genes in the egg apparatus of maize and other cereals to induce e.g. parthenogenesis as a major component of the apomixis trait.

Genomic Southern blots suggested the presence of *ZmEA1* homologous genes in maize and other cereals, but no obvious homologs in dicotyledonous plant species. It would be interesting to investigate if any of these plays a conserved role in fertilisation or if they have evolved different functions. One of the two rice homologous genes, *OsEAL2*, is lowly expressed in callus, young leaf, root and panicle based on the presence of 5 ESTs in the public database. The other homolog, *OsEAL1*, represents a hypothetical protein and might represent the functional ortholog of *ZmEA1*. On the evolutionary level, it is interesting to note that *ZmEA1* appears to be present only in the Gramineae and perhaps other monocotyledonous plant species and not in the dicotyledonous plants. These observations suggest a gramineae-specific evolution and function for *EA1*-like genes. They might have a gramineae-specific cell identity function or be involved in pollen tube guidance. To prove these hypotheses more detailed investigations have to be done.

4.3 ZmEA1:GFP is secreted into the filiform apparatus and generates a gradient within the micropylar region

A ZmEA1:GFP C-terminal fusion protein was secreted in a floral developmental stage dependent manner from the egg apparatus towards the filiform apparatus and appeared in nucellus cell layers at the micropylar region of unfertilised ovules. GFP signals were faint in unfertilised young ovules, shortly after silk emergence, and increased in unfertilised mature ovules, after silk elongation (>10 cm), correlating well with maturation of the egg apparatus during the female receptivity period in maize (Mól *et al.*, 2000).

The production of *ZmEA1* appears to be high at least at the transcriptional level representing more than 3% of the total number of ESTs isolated from the egg cell. Based on the *in situ* hybridisation data (Cordts, 2000), expression would be the same or even higher in the synergids. Such transcriptional activity could be required for high protein production for secretion into the filiform apparatus, either as a component of this structure or to generate a protein gradient from the egg apparatus. The encoded ZmEA1 protein is probably secreted via the secretory pathway towards the cell wall material of the synergids (which is mainly consisting of the filiform apparatus), from

where a gradient is established in the cell walls of nucellus cells forming the micropyle. Many secretory vesicles have been observed at the micropylar region of synergids that might carry relatively large amounts of the ZmEA1 peptide (Diboll, 1968). To identify the subcellular localisation of the predicted ZmEA1 peptide, onion epidermal cells were used for a transient transformation assay. Onion epidermal cells are ideal for imaging cellular structures and observing GFP fusion proteins, because they are large, transparent, have little autofluorescence and are present in a single cell layer (Scott *et al*., 1999). In contrast, the maize embryo sac is deeply embedded in the ovule tissues. The embryo sac cells could be isolated with enzymatic treatment, but subcellular structures like transvacuolar strands, organelles, etc., could not be clearly identified by microscopy. Onion epidermal cells expressing a ZmEA1:GFP C-terminal fusion protein showed that the fusion protein is localised in the endoplasmic reticulum surrounding the nucleus, in transvacuolar strands as well as in large vesicles accumulating at the plasmamembrane supporting the hypothesis that ZmEA1 is secreted in high amounts to the filiform apparatus via the secretory pathway.

Ishikawa suggested already in 1918 that the filiform apparatus is required for pollen tube attraction, and in 1964, van der Pluijm postulated that chemotropic substances are produced by the synergids. These hypotheses were only substantiated when Higashiyama and colleagues (2001) demonstrated the existence of a short-range diffusible attraction signals by cell ablation using lasers in the *in vitro Torenia* system. They suggested that the attractant diffuses some 100-200 µm around the egg apparatus. It was shown as well that a single synergid cell was necessary and sufficient to produce the attraction signal, although the second synergid cell enhances it. Synergid cells appear to have metabolic functions, including protein synthesis and secretion, which may be involved in the synthesis of the pollen-tube attraction signal in higher plants (Higashiyama, 2002). *ZmEA1* is also expressed in the egg cell. Nevertheless, the *ZmEA1* encoding transcript might be transported via plasmodesmata to the synergid cells to enhance the transcript abundance and thus the amount of the ZmEA1 protein. Plasmodesmata do not exist in the outer wall of the megagametophyte, but they are present within the cells of the megagametophyte (Diboll, 1968).

Confocal laser scanning microscopy showed a cell wall localised ZmEA1:GFP fusion protein from the egg apparatus (highest signal) to a restricted number of micropylar nucellar cells. The fusion protein accumulated at the surface of nucellus cells of the micropyle within an area of around 150 µm. This may suggests a possible gradient

creation based on passive diffusion of the protein. However, the diffusion was restricted only to the micropylar region suggesting some negative regulation of the transport in the surrounding integuments and the adjacent nucellar cells. The relatively conserved C-terminus identified in ZmEA1 and its rice homologs suggests that this might be the biologically active part of the peptide. This region is relatively large and thus may limit diffusion over long distances. It is a theoretical possibility that the artificial GFP fusion might hinder the diffusion of ZmEA1:GFP into further areas of the apoplast.

After fertilisation, the ZmEA1:GFP fusion peptide was no longer detectable in maize, suggesting active degradation of the ZmEA1 peptide after fertilisation. This correlates with the expression data showing that *ZmEA1* is down regulated after fertilisation (Cordts, 2000). In *Torenia fournieri*, it was shown that the embryo sac no longer attracts the pollen tube after fertilisation, despite the persistence of one synergid cell (Higashiyama *et al.*, 2001). It is supposed that the cessation of pollen tube attraction to the fertilised embryo sac might be involved in preventing polyspermy during the fertilisation in higher plants, but it is still not known whether the attraction signal from the synergid is no longer secreted after fertilisation or whether a repulsive signal or a decomposing molecule affects pollen tube attraction by fertilised female gametophytes (Higashiyama *et al.*, 2003). Certainly, by being the first FG structure reacting to pollination, via cell death, synergids must have a role in the rapid down regulation of the gene in all cells producing the guidance signal. The rapid loss of ZmEA1:GFP signal after fertilisation may suggest proteolysis as another regulatory pathway along with rapid transcriptional regulation of PT guidance to optimise pollen fertilisation by not attracting fertile pollen tubes to already fertilised FGs or to prevent polyspermy. All these data suggest that the egg-apparatus specific gene *ZmEA1* may encode for a signalling protein that can play an important role in pollen tube guidance in maize. In order to prove this hypothesis, further experiments were performed.

4.4 ZmEA1 peptide is required for close range micropylar pollen tube guidance in maize

Since the chromosomal location of *ZmEA1* was not linked to any phenotypic markers, functional analysis of *ZmEA1* loss of activity was done via a transgenic approach in maize. RNA interference (RNAi) and antisense (AS) lines were generated after transforming inbred (A188) or hybrid (A188 x H99) immature embryos. Transformation

efficiency using inbred embryos was 2.6 times lower than using hybrid embryos, thus confirming the literature about a low efficiency rate when transformation is performed with embryos from a pure inbred line (Brettschneider *et al.*, 1997). Transgenic lines obtained had multiple complete and/or incomplete transgene integrations (≥2 to ≥10). Therefore, for the future, one can use *Agrobacterium*-mediated transformation to produce transformants of maize inbred A188 from immature embryos, with a higher efficiency rate (5-30%) compared to biolistic transformation (2-4%), and a low number of transgene integrations (1-3) (Ishida *et al.*, 1996).

For both RNAi and AS-approaches, most transgenic T0-lines with complete transgene integrations showed a significant reduction of seed set (0-75%) upon selfing, but full seed set (100%) after crossing pollen to wild type (wt) plants. This indicated that the female gametophyte, but not the male gametophyte (MG, pollen) is affected, as it was expected. The four RNAi transgenic lines lacking complete transgene integrations displayed a seed set comparable to wt (95-100%). The female sterility effect was especially strong when inbred embryos (A188) have been used. The *ZmEA1* gene was original isolated from the maize inbred line A188 and has a homologue in the inbred line H99. It might be possible that there are differences in the sequences of both homologues genes and thus, *ZmEA1* gene was stronger down regulated in the plants originating from A188 inbred embryos than in those from embryos of A188xH99 crosses. It might also be that the *ZmEA1* gene is higher expressed and thus more difficult to down regulate in the egg apparatus of hybrids originating plants. The results of the single cell Q RT-PCR analysis supported this hypothesis.

Although there is little variation between plants with the same integration pattern, the strong variation observed among independent T0- and T1-lines is probably due to the segregation of the multiple transgenes in multiple chromosomal loci. It is also possible that some integrations do not completely remove *ZmEA1* activity. However, the phenotype observed in most RNAi-plants from the T0 generation (reduced seed set after selfing or out-crossing with wt pollen, but full seed set after crossing the pollen to wt plants) was also maintained in the T1 generation indicating again that the FG, but not the MG is affected. The progeny of one RNAi line showed pleiotropic phenotypes (female sterility, male sterility and dwarfism) in T1-plants, perhaps due to different causes. It is possible that full-copies and/or partial fragments of the transgene were integrated in genome loci disrupting the expression of genes important for house

keeping functions and development. The T1-plants were generated in the winter season and the light requirement of maize might not have been satisfactory.

Microscopy studies showed that the fertilisation efficiency in the RNAi plants (40-55%) was significantly reduced compared to the one in the wt plants (82%), although ovary, ovule and FG development was not affected. In the RNAi plants, pollen tubes (PTs) lost their way towards 45-60% FGs. The majority of PTs did not enter the micropyle although pollen tubes were visible at the surface of the inner integument of all ovules analysed. In most cases pollen tubes grew at random directions at the surface of the inner integument. These types of loss of PT guidance were never observed in wt ovules. Here, one PT per ovule stopped growth abruptly at the micropyle to penetrate the nucellus tissue and grow towards the egg apparatus for releasing its content into the receptive synergid. These results demonstrate that ZmEA1 functions as a short-range signalling protein required for micropylar pollen tube guidance in maize.

Interestingly, most of the current knowledge about PT guidance mediated by the FG, with the exception of an *in vitro* system in *Torenia fournieri*, that contains naked embryo sacs (Higashiyama *et al*., 2001), has been derived from the dicot model plant *Arabidopsis thaliana*. PT guidance among plant species is likely to be quite different as reproductive structures are morphologically and physiologically diverse. For example, in *Arabidopsis* each ovary contains some 50 ovules, while ovaries of cereals contain a single ovule. In *Arabidopsis* only one PT usually grows toward each ovule (Hülskamp 1995; Shimizu and Okada, 2000), while many PTs were observed at the surface of maize ovules. A cell file of 5-6 nucellus cell layers forms part of the micropyle in maize and other cereals, but only 1-2 cell layers are visible in *Arabidopsis*, where both integuments generate a micropylar channel.

Using various gametophytic and sporophytic mutants in *Arabidopsis thaliana*, Hülskamp *et al.* (1995) and Ray *et al.* (1997) showed that PTs of mutants with incompletely formed ovules or lacking FGs arrive at the surface of the placenta without entering the ovule. They also concluded that the distance of attraction was not exceeding ~200 µm. Additionally, in the *Arabidopsis* female gametophyte *magatama* mutants containing immature FGs, the pollen tubes grow normally along the funiculus but lose their way within 100 µm distance of the micropyle (Shimizu and Okada, 2000). Thus, guidance by the FG in *Arabidopsis thaliana* was subdivided in funicular and micropylar guidance. Shimizu and Okada (2000) suggested that the egg apparatus, composed of synergid and egg cells, is responsible for funiculus guidance, and possibly also for micropyle

guidance. In *Torenia fournieri*, Higashiyama *et al.* (2001) identified the synergids as the source of producing short range diffusible attraction signal(s), about 100-200 µm around the egg apparatus. These signalling molecules seem to be species specific and have been suggested to represent peptides secreted by the synergids (Higashiyama *et al.*, 2003). ZmEA1 represents the first peptide/small protein, which was identified that supports all these hypotheses. *ZmEA1* is highly expressed in the egg apparatus, is secreted at the micropylar region generating a detectable gradient of 40-50 µm and down regulation by RNAi resulted in loss of pollen tube guidance only at the micropylar region.

Higashiyama *et al.* (2003) reported that pollen tubes of related species did not respond to the *Torenia fournieri* attraction signal. A similar observation was made conducting interspecific crosses using *Arabidopsis* and other Brassicaceaes where PTs grew normally through the transmitting tissue but rarely arrived at the funiculus and did not enter the micropyle (Shimizu and Okada, 2000). All these observations suggest the existence of species-specific guidance signals rather than general signals such as GABA (Palanivelu *et al.*, 2003) or NO (nitric oxide, Prado *et al.*, 2004). The occurrence of species-specific peptides/small proteins for pollen tube guidance might be the explanation for these findings. *ZmEA1* and related genes were identified in cereals, but not in dicotyledonous species. This might also be one explanation, that wide crosses involving successful pollen tube guidance and fertilisation are possible within genera of the Gramineae, but not between species spanning wider taxonomic boundaries (Sharma, 1995).

A ZmEA1:GFP fusion protein accumulated at the surface of the micropyle, but not at the integuments, indicating that (a) further signal(s) are generated either by the FG and/or the integuments to generate long distance attracting signals. Smaller molecules including oligopeptides consisting of a few amino acids and molecules such GABA and NO might be candidates for long distance, but species-unspecific PT guidance. This may imply that, in maize, pollen tubes are first attracted in relatively high numbers by the long range signal to the proximity of the micropyle and that random growth/contact of one of these PT over the protruding nucellus in the micropylar region is needed to sense the close range ZmEA1 signal. The results obtained during this work are consistent with this hypothesis.

After fertilisation, the ZmEA1:GFP fusion peptide was no longer detectable, suggesting active degradation of the ZmEA1 peptide. This might play a role in optimising the

fertilisation process by not attracting fertile pollen tubes to already fertilised FGs or to prevent polyspermy. Huck *et al.* (2003) have shown that two or more PTs can enter mutant FGs in *Arabidopsis feronia* mutants. Apparently, in the *feronia* FG, the capacity to produce attractant is not lost immediately, which would lead to the attraction of multiple pollen tubes. It has been shown also that the fertilised FG of *Torenia fournieri* no longer attracts pollen tubes, although one synergid cell still exists after pollen-tube discharge (Higashiyama *et al.*, 2001). Degradation of FG specific pollen tube attractant(s) seems to be a prerequisite to prevent arrival of supernumerary PTs and, subsequently, polyspermy. The results described in this work are in accordance with these assumptions, as the *ZmEA1* gene is down regulated after fertilisation and the fusion protein disappeared.

4.5 Outlook

The results of this work have shown that *ZmEA1* encodes for a small protein that was shown to be a short-distance signalling protein required for micropylar pollen tube guidance during the fertilisation process in maize. Based on genomic Southern blots, homologous genes were also present as single copy genes in other cereals (barley and *Tripsacum dactyloides*) with the exception of rice where two homologous genes were identified. An aim for the future will be the isolation of these genes from other grasses, but also from other maize inbred lines. The two rice homologous genes should be down regulated in order to see if any of these plays a conserved role in fertilisation or in pollen tube guidance or if they have evolved into a different pathway. The identification of further pollen tube attractants should not only help to understand many of the outstanding issues in plant reproductive biology, but may also be used for future plant breeding to overcome crossing barriers and to allow extremely wide hybridisation between plant genera, which cannot be crossed today.

The nature of the mature ZmEA1 peptide is not known and it is very likely that the protein is modified after translation. A cell wall protein extract could be obtained from the microdissected ovule region containing the micropyle, separated on a 2D gel and compared with another cell wall protein extracts from leaves, for example. Finally, the spots obtained could be analysed by MALDI-TOF (Matrix Assisted Laser Desorption Ionisation TOF (Time Of Flight)) mass spectrometry. A recombinant ZmEA1 protein could be generated using, for example, a yeast expression system, such as *Pichea*

pastoris. Yeasts dispose of an eukaryotic protein secretion machinery and could complete posttranslational modifications such as proteolitic processing, O-glycosilation, etc. (Eckart and Bussineau, 1996; Higgins and Cregg, 1998).

The report of a maize specific peptide required exclusively for micropylar guidance provides further evidence, that PT guidance is a complex biological process involving extensive cell-cell communication between the MG and sporophytic tissues (stigma, style, ovary, integuments, micropyle) as well as between MG and FG. Thus, a very important task is the identification of a ZmEA1 receptor in the plasma membrane of the pollen tube. Finding interaction partners could be done using, for example, a two yeast hybrid system. Many candidates for pollen-surface receptors have been identified already in few species such as tomato and *Arabidopsis* (Tang *et al.*, 2002; Honys and Twell, 2003) and are likely to be present also in pollen tubes tips of cereals.

5 Summary

The development and function of the female gametophyte (embryo sac) as well as double fertilisation are fundamental issues in plant reproductive biology. Although extensively studied at the cytological level, little is known about the molecular and genetic processes controlling embryo sac development and function. For example, the molecules secreted by the female gametophyte to attract the male gametophyte are unknown.

The aim of this work was to identify genes highly and specifically expressed in the female gametophyte and to perform functional analysis with a gene encoding a possible secreted protein as a ligand for cell-cell communication processes. From 988 maize egg cells ESTs generated during this thesis, it was selected the novel and second highly expressed female gametophyte-specific gene *ZmEA1* (*Zea mays Egg Apparatus1*). RT-PCR analyses with different maize tissues showed that *ZmEA1* is exclusively expressed in the maize egg apparatus. The full length *ZmEA1* cDNA was cloned by 5' RACE and the transcription start point (TSP) was confirmed by single cell (SC) RT-PCR using isolated egg cells. *ZmEA1* does not contain introns and was mapped to the long arm of chromosome 7, a region lacking phenotypic markers. *ZmEA1* encodes a small protein of 94 amino acids with a predicted transmembrane domain. Based on hybridisations, homologous genes were present as single copy in other cereals (barley and *Tripsacum dactyloides*) with the exception of rice where two homologs were identified.

1,463 bp DNA upstream of the *ZmEA1* TSP was isolated as the *ZmEA1* promoter (*ZmEA1*p). The specificity and functionality of the *ZmEA1* promoter was analysed in transgenic maize and rice plants containing the *GUS* coding sequence under the control of the *ZmEA1* promoter. Using histochemical GUS-assays, GUS activity was exclusively detected in the egg apparatus of unpollinated mature ovules in three independent functional *ZmEA1*p*::GUS* transgenic maize lines. GUS transcripts were also detected by RT-PCR in pistils of three different transgenic rice lines demonstrating that the *ZmEA1* promoter is also functional in rice.

Subcellular localisation of the predicted ZmEA1 peptide was studied in transiently transformed onion epidermal cells expressing a ZmEA1:GFP fusion protein under the control of the maize constitutive ubiquitin promoter. The fusion protein was localised in the endoplasmic reticulum surrounding the nucleus, in transvacuolar strands as well as in large vesicles accumulating at the plasmamembrane. To study ZmEA1 protein localisation in maize, transgenic maize lines expressing a ZmEA1:GFP fusion protein

under the control of the *ZmEA1* promoter were generated. The fusion protein was secreted in a floral developmental stage dependent manner from the egg apparatus towards the filiform apparatus and appeared in nucellus cell layers at the micropylar region of unfertilised ovules in four independent transgenic lines. GFP signals were faint in unfertilised young ovules, shortly after silk emergence and increased in unfertilised mature ovules (silk elongation $\rightarrow$10 cm) generating a gradient from the filiform apparatus towards the surface of the micropyle. Confocal laser scanning microscopy (CLSM) observations confirmed a presence of a cell wall localised ZmEA1:GFP fusion protein within the nucellus cells of the micropyle. 24 h after *in vitro* pollination (around 18 h after fertilisation), signals of the fusion protein were no longer detectable in the samples analysed.

RNA interference (RNAi) and antisense (AS) approaches have been applied to study the function of *ZmEA1* in maize. Most transgenic lines with complete transgene integrations showed a significant reduction of seed set upon selfing (0-75%), but full seed set (100%) after crossing pollen to wild type plants indicating that the female gametophyte but not the male gametophyte is affected. Four RNAi lines with incomplete transgene integrations displayed a seed set comparable to the wild type. Single cell Q RT-PCR analysis was performed with isolated egg cells from a T1 RNAi transgenic plant to quantify *ZmEA1* transcript amounts. The *ZmEA1* gene was significantly down regulated in 5 of 9 egg cells from the RNAi line showing ovule sterility, but none of the egg cells displayed a complete down-regulation. *In vitro* pollination studies showed that the fertilisation efficiency of RNAi plants was significantly reduced (40-55%) compared to wild type plants (82%), although ovary, ovule and female gametophyte development appeared not to be affected. Detailed microscopic analyses showed that pollen tubes were visible at the surface of the inner integument of all transgenic ovules analysed, but the majority of them did not enter the micropyle and in most cases grew at random directions at the surface of the inner integument. These types of loss of pollen tube guidance were never observed in wild type ovules. Based on observed phenotypes, it can be concluded that reduction of *ZmEA1* activity in the egg apparatus affects micropylar, but not long range pollen tube guidance in about half of the ovules.

In summary, *ZmEA1* represents the first gene discovered to be specifically expressed in the egg apparatus of a plant species and encodes for a short-range signalling protein required for micropylar pollen tube guidance in maize. Thus, ZmEA1 is also the first female gametophyte pollen tube attractant discovered so far.

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