The Requirement of WHIRLY1 for Embryogenesis is Dependent on Genetic Background in Maize

ZHANG, Yafeng

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

Biology

The Chinese University of Hong Kong

September 2013

Thesis/Assessment Committee

Professor **Hon-Ming Lam**	(Chair)
Professor **Bao-Cai Tan**	(Thesis Supervisor)
Professor **Jun-Xian He**	(Committee Member)
Professor **Yi-Ji Xia**	(External Examiner)

Abstract of thesis entitled:

The Requirement of WHIRLY1 for Embryogenesis is Dependent on Genetic Background in Maize

Submitted by **ZHANG Yafeng**

For the degree of **Doctor of Philosophy in Biology**

at The Chinese University of Hong Kong in September 2013

Plastid gene expression is essential to embryogenesis in higher plants, but the underlying mechanism is obscure. Through molecular characterization of an *embryo defective 16* (*emb16*) locus, we find that the requirement of plastid translation for embryogenesis is dependent on the genetic background in maize (*Zea mays*). The *emb16* mutation arrests embryogenesis at transition stage and allows the endosperm to develop largely normally. Molecular cloning reveals that *Emb16* encodes WHIRLY1 (WHY1), a DNA/RNA binding protein that is required for genome stability and ribosome formation in plastids. Interestingly, the previous *why1* mutant alleles (*why1-1* and *why1-2*) do not affect embryogenesis, only conditions albino seedlings. The *emb16* allele of *why1* mutation is in the W22 genetic background. Crosses between *emb16* and *why1-1* heterozygotes resulted in both defective embryos and albino seedlings in the F1 progeny. Introgression of the *emb16* allele from W22 into A188, B73, Mo17, Oh51a and the *why1-1* genetic backgrounds yielded both defective embryos and albino seedlings. The embryo development in *emb16* mutant went beyond the transition stage and was arrested in a consecutive manner from the transition stage to seed maturity in the F2s of the first backcross generation. Similar results were obtained with two other *emb* mutants (*emb12* and *emb14*) that are impaired in plastid protein translation process. These results indicate that the

requirement of plastid translation for embryogenesis is dependent on genetic backgrounds and regulated by multiple genetic loci that are present differently between W22 and A188, B73, Mo17, or Oh51a inbred. Thus, this study implies the presence of a mechanism underlying embryo lethality suppression in maize.

玉米胚胎發育是否需要 WHIRLY1 蛋白均定於其所在的遺傳背景

摘要

高等植物的胚胎發育需要葉綠體基因的表達，但其機理尚不清楚。通過對玉米 *Embryo defective 16* (*Emb16*) 位點的分子克隆和特征分析，我們發現胚胎發育是否需要葉綠體基因的表達是由其所在遺傳背景決定的。*emb16* 是一個胚胎發育缺陷型突變體。它的胚胎發育受阻於過渡期，但是胚乳發育沒有受到明顯影響。對 *Emb16* 位點的分子克隆結果表明，*Emb16* 基因表達 WHIRLY1（WHY1）蛋白。WHY1 是一個 DAN/RNA 結合蛋白，負責葉綠體基因組的穩定和核糖體的形成。之前的研究表明 *why1* 突變體（*why1-1* 和 *why1-2*）的表型為白化苗或者黃化苗，胚胎發育正常。*emb16* 突變在 W22 自交系遺傳背景中，而 *why1-1* 和 *why1-2* 的遺傳背景未有報道。將 *emb16* 和 *why1-1* 雜交，雜交一代群體中既有胚胎缺陷型突變體又有白化苗突變體。將在 W22 背景的 *emb16* 雜合體與 A188，B73，Mo17 及 Oh51a 自交系雜交，在 F2 中既有胚胎發育缺陷型突變體又有白化苗突變體。*emb12* 和 *emb14* 是兩個與 *emb16* 相似的胚胎發育突變體。EMB12 和 EMB14 蛋白也參與了葉綠體基因組的翻譯。同樣地，將 emb12 和 emb14 在 W22 背景的雜合體與 A188，B73 和 Mo17 自交系雜交，在 F2 代中既有胚胎發育缺陷型突變體，又有白化苗突變體。此外，在这些 F2 代中，三個突變體的胚胎發育都通過了過渡期，並以一個連續的方式受阻於過渡期與完全發育胚胎之間。這些結果表明，胚胎發育的過程是否需要葉綠體基因組表達是由一些未知的遺傳因子決定。這些遺傳因子由多個遺傳位點控制，而且它們在 W22 與其他玉米自交系的表达存在差異。正是由於這種差異導致了 *why1*，*emb12* 和 *emb14* 在不同遺傳背景下表型的差異。同时，這些研究結果揭示了一條新的葉綠體基因翻譯調節胚胎發育的途徑。這條途徑與葉綠體翻譯的過程有關，卻與葉綠體基因沒有關系。

Declaration

I hereby declare that I am the sole author of the thesis.

I authorize The Chinese University of Hong Kong to lend this thesis to other institutions or individuals for the purpose of scholarly research.

I further authorize The Chinese University of Hong Kong to reproduce the thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

ZHANG, Yafeng

Acknowledgements

I would like to express my sincere gratitude to my supervisor Prof. B.C. Tan, not only for the interesting project he gave me, but also for his supervision throughout the four years of my Ph.D. study. He teaches me a lot in plant molecular biology, and usually shares with me his new ideas. His recognition and encouragement on my experiments make me progress step by step. The most important is his critical attitude and open mind thinking in doing science. All of these will be helpful for me in my future work.

I am grateful to the members of my graduate committee, Prof. H.M. Lam and Prof. J.X. He for their academic advices and encouragement in these years. I am also grateful to Prof. Y.J. Xia (Hong Kong Baptist University) for being the external examiner of my thesis defense, and Prof. W.C. Yu for being the marker of my prelim examination.

Special thanks are given to Prof. Tan for his work on the co-segregation analysis of *emb16* mutant, and my labmates, Dr. Yun Shen for her technical assistance on TEM and subcellular localization of ACR1 protein, Dr. Yujun Liu for her help in 2-D gel electrophoresis, and Mr. Mingming Hou for his effort in the localization experiment of WHY1 protein. I would like to thank the current and past labmates in SC LG90, Miss Yi Chen, Mr. Yanzhuo Yang, Mr. Jerry Xiaojie Li, Mr. Zhihui Xiu, Dr. Xiaomin Wang, Dr. Feng Sun, Miss Angle Wing Ho Sze, Dr. Cuiling Li (Shandong University), Mr. Zongliang Chen (The China Agricultural University), Prof. Yuanlin Duan (Fujian Agriculture and Forestry University), Prof. Xueping Li (South China Agriculture University).

Besides, thanks are also due to the staff of the School of Life Sciences, especially, Mr. Freddie Kwok and Miss. Bondy Lai Shui Ho, and to my fellows, especially Dr. Steffi Xiaohang Long. I am indebted to Steffi for her sincere help in the 2-D gel electrophoresis and MS/MS experiments.

Finally, I would like to express my deep appreciation to my family, my wife, my mother and father. Without their understanding, support and encouragement, this thesis would not be possible.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACCase	acetyl-CoA carboxylase
ACR	ACT domain repeat protein
AD	arbitrary degenerated primers
ATP	adenosine triphosphate
BC	biotin carboxylase
BCCP	biotin carboxyl carrier protein
BLAST	Basic local alignment search tool
CAF2	CRS2-associated factor 2
clpP1	caseinolytic protease P1
CoA	coenzyme A
CT	carboxyl transferase
DAG	diacylglycerol
DAP	days after pollination
dek	defective kernel
DGDG	digalactosyldiacylglycerol
emb	embryo defective
emp	empty pericarp
g	gram
RCF	relative *centrifugal force*
GFP	green fluorence protein
Glc	glucose
H3	histone 3
He-ACCase	heteromeric acetyl-CoA carboxylase
Ho-ACCase	homomeric acetyl-CoA carboxylase
hr	hour
ij	iojap

kb	kilo basepair
kD	kilo dalton
KP1	kinesin-like protein 1
M	molar
MGD1	MGDG Synthase 1
MGDG	monogalactosyldiacylglycerol
min	minutes
ml	mililiter
mm	milimeter
mM	milimolar
MS	Murashige and Skoog
Mu	Mutator
O5	Opaque5
PCR	Polymerase chain reaction
PPR	pentatricopeptide repeat protein
PRP	plastid ribosomal protein
PSI-BLAST	Position-Specific Iterated BLAST
RBCL	RUBISCO large subunit
rRNA	ribosomal RNA
RT-PCR	reverse transcriptional PCR
smk	small kernel
TAIL	Thermal asymmetric interlace PCR
TEM	transmission electron microscopy
THA8	thylakoid assembly 8
TIR	terminal inverted repeat
tRNA	transfer RNA
UPD	Uridine diphosphate
UV	ultraviolet
Why1	Whirly1

WT	wild-type
µm	micrometer

Chapter 1 Introduction

Maize (*Zea mays*) is an important crop around the world. First of all, it is an important renewable resource for food, fodder, and industrial raw materials. Secondly, it is a model organism for basic research. Several attributes of maize, including a vast collection of mutant stocks, large heterochromatic chromosomes, extensive nucleotide diversity, and genic colinearity within related grasses, have positioned it as a centerpiece for genetic, cytogenetic, and genomic research (Strable and Scanlon, 2009). Given its advantage and significance, a lot of attentions have been paid to the studies on maize and the production of maize is increasing year by year (FAO). In 2011, the world production of maize reached 880 million tons (Figure 1.1; FAO, 2011), which was higher than other major crops including rice (*Oryza sativa*, 723 million tons), wheat (*Triticum* spp, 704 million tons) and barley (*Hordeum vulgare*, 134 million tons).

1.1 Seed development in angiosperm

In angiosperms, a seed consists of three genetically distinct constituents: (1) the diploid embryo, (2) the triploid endosperm, and (3) the seed coat derived from the maternal tissue of the ovule. The development of a seed is initiated with a double fertilization process, involving the fusion of the egg cell with a sperm nucleus and the central nuclei with a second sperm nucleus (for an overview see Bennetzen and Hake, 2009). After fertilization, the fertilized egg cell, i.e. zygote, follows a pattern in the first round division, which is asymmetric and oriented perpendicular to the micorpylar/chalazal axis of the embryo sac. This results in a small apical and a large basal cells, which have different fates. The apical cell develops into the embryo proper, and the basal cell forms the suspensor. After several rounds of division, the zygote goes to the embryo transition stage, and eventually forms a basic plant body including shoot and root meristems. Synchronously with embryo development, the development of endosperm begins with the repeatedly division of the fertilized central

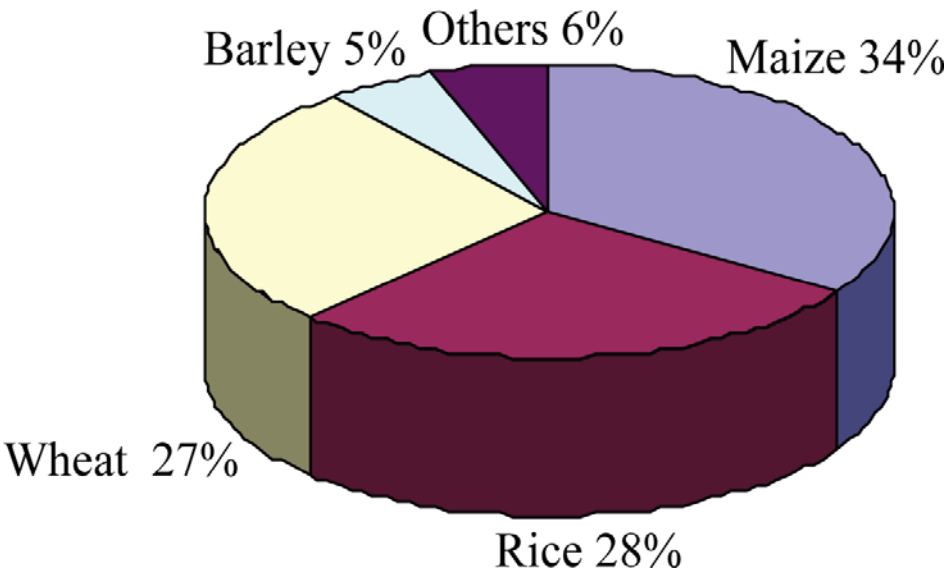


Figure 1.1 The production of world major cereal in 2011

nuclei without cell wall formation (Olsen, 2004). This results in a characteristic coenocyte-stage endosperm. Via the formation of radial microtubule system and alveolation, the endosperm coenocyte is cellularized. After being completely cellularized, the endosperm undergoes cell fate specification and differentiation to form starchy endosperm, aleurone, transfer cells, and the cells of the embryo surrounding region. In dicots, the completely cellularized endosperm is consumed during seed maturation, leaving only the peripheral aleurone-like cell layer. By contrast, the mature seed of a monocot bears a large endosperm, which takes most volume and weight of a seed.

1.2 Plastids play an essential role in plant embryogenesis

To study the mechanism underlying the complex processes of seed development, many seed mutants have been isolated, mainly in maize and *Arabidopsis* (Meinke *et al*., 2008; McElver *et al*., 2001; Tzafrir *et al*., 2004; McCarty *et al*., 2005; Clark and Sheridan, 1991). These seed mutants are general termed as *embryo defective* (*emb*) in *Arabidopsis*, which is comprised of mutants with embryo lethality or aberrant seedling (Meinke *et al*, 2008; Bryant *et al*., 2011). Due to the different seed structure between *Arabidopsis* and maize, such seed mutants in maize are classified into *defective kernel* (*dek*), *empty pericarp* (*emp*), *small kernel* (*smk*), and *embryo defective* (*emb*) based on the impact on the embryo and endosperm. Unlike *Arabidopsis*, the same term *emb* in maize describes seed mutants with specific arrest in embryo development and without significant deleterious impact on endosperm development (Clark and Sheridan, 1991). By contrast, the other three subclasses are defective in both embryo and endosperm development (McCarty *et al*., 2005). At present, there is no report on the maize seed mutation which specifically arrests endosperm development, but allows embryo development normally. Comparing the genes essential for seed mutants between *Arabidopsis* and maize, one can expect that the number of *Emb* genes in *Arabidopsis* is bigger than that in maize; the *Arabidopsis Emb* gene may be orthologous to a maize *Dek*, *Emp*, or *Smk* gene, which means a

mutation in an *Arabidopsis Emb* gene may be defective in both embryo and endosperm development in maize.

Through both forward and reverse genetic analyses on the *emb* mutants in *Arabidopsis*, many cellular processes are revealed to be essential for embryogenesis, such as chromatin remodeling, DNA replication, RNA synthesis and transcriptional regulation, protein translation and degradation, and lipid synthesis (Tzafrir *et al*., 2004). Compared with genes with other knockout phenotypes, *Emb* genes are enriched for basal cellular functions, deficient in transcription factors and signaling components, and a fraction of them encoded proteins targeted to plastids (Tzafrir *et al*., 2004). Recent studies on the role of plastid-localized proteins in embryogenesis showed that about 30% of EMB proteins essential for embryogenesis take functions in plastids (Hsu *et al*., 2010; Bryant *et al*., 2011), thus revealing an important role of plastids in the embryogenesis of *Arabidopsis*.

Plastid is an organelle vital to all photosynthetic and some non-photosynthetic eukaryotes. It is the site for photosynthesis, and houses many metabolic processes such as the biosynthesis and accumulation of starch, fatty acids, amino acids, and various terpenoids, including carotenoids and precursors to gibberellins (Seo and Koshiba, 2003; DellaPenna and Pogson, 2006; Neuhaus and Emes, 2000; Yamaguchi and Kamiya, 2000). Genetically, plastid is a semi-autonomous cellular organelle that has its own genome and gene expression machinery (Sugiura, 1992). It is derived from a cyanobacterium-like ancestor that invaded or was engulfed by a eukaryotic host (Dyall *et al*., 2004). During the endosymbiotic evolution, most of its genes were either lost or transferred to the host nuclear genome. The resultant plastid genome contains about 111 genes, which encode about 70 proteins and can be classified into three main categories: genes for the photosynthetic apparatus, those for the transcription/translation system, and those related to biosynthesis (Wakasugi *et al*., 2001). In addition to genes with known functions, the plastid genome contains a

number of open reading frames with unknown functions, e.g. *ycf1* and *ycf2* (Wakasugi *et al*., 1998; Sato *et al*., 1999).

The functional analyses on the plastid-localized EMB proteins in *Arabidopsis* showed that eliminating of the biosynthesis of amino acid, vitamin, nucleotide, or lipid acid in plastids and interfering with plastid translation machinery often result in embryo lethality, but disabling the photosynthetic machinery or reducing the levels of chlorophyll, carotenoids, or terpenoids leads instead to reduced pigmentation and altered physiology but embryo viable (Bryant *et al*., 2011). For example, loss of function in proteins essential plastid protein translation, ***e.g.*** plastid ribosomal proteins (PRPs) and several pentatricopeptide repeat proteins (PPRs) cause embryogenesis arrest (Li *et al*., 2011; Romani *et al*. 2012; Cushing *et al*., 2005). In plastid, the galactolipid, monogalactosyldiacylglycerol (MGDG) is synthesized by the MGDG Synthase 1 (MGD1). Loss of function in MGD1 protein leads to embryo lethality as well (Kobayashi *et al*., 2007).

In consistent with *Arabidopsis*, disruptions of the biosynthetic functions and defects in plastid translation machinery also lead to embryo lethality in maize. *Opaque5* (*O5*) gene encodes the MGD1 in maize (Myers et al., 2011). The *o5* mutant is defective in the biosynthesis of galactolipid, and aborted in both embryo and endosperm development. *lem1* and *emb8516* are two *emb* mutants in maize, which are defective in embryo development, but allow endosperm development normally (Ma and Dooner, 2004; Magnard *et al*., 2004). The two genes encode the plastid *RPS9* (*Lem1*) and *RPL35a* (*Emb8516*), respectively. *emb12* and *emb14* are two another *emb* mutants, and *Emb12* and *Emb14* genes respectively encode the translation initiation factor 3 and an YqeH homolog in plastids, both of which are required for the formation of translation machinery (Shen *et al*., 2013; Li C. and Tan, B.C., unpublished data).

In addition to *Arabidopsis* and maize, the *defective chloroplast and leaf* mutant in tomato (*Solanum lycopersicum*) is defective in the processing of plastid 4.5S rRNA,

and shows embryogenesis arrest at the globular stage, which suggests the normal functional plastid translation is also essential for embryogenesis in tomato (Bellaoui *et al*., 2003).

1.3 The mechanism that plastid translation regulates embryogenesis

The recent issue on the regulation of embryogenesis by plastids remaining to be addressed concerns the identity of the chloroplast genes required for embryogenesis. If interfering with plastid translation results in embryo lethality, there must be one or more chloroplast genes whose influence extends beyond the plastid compartment and are also essential for cell viability.

So far, the essential nature of several plastid genes in cell viability has been demonstrated in tobacco (*Nicotiana tabacum*; Drescher *et al*., 2000; Shikanai *et al*., 2001; Kode *et al*., 2005). To study the function of a plastid gene, the chloroplast transformation strategy is adopted (for an overview see Drescher *et al*., 2000). First, the knock-out alleles for a certain gene or open reading frame in plastid genome are constructed by deletional or insertional mutagenesis. Second, the mutant alleles are integrated into the plastid genome where they replace the endogenous intact allele by homologous recombination. If the replaced gene is not essential for cell viability, a homoplastomic state of the transformed genome is achieved by repeated cycles of plant regeneration under highly selective pressure (usually selected by the antibiotic of spectinomycin). This selective pressure favors high expression of the transplastome and thereby drives the random genome sorting towards the accumulation of transformed genomes. Thus, the resulting homoplastomic transplastomic plants will entirely lack the wild-type (WT) allele and, hence, will reveal the phenotype of plants deficient for the gene product encoded by the open reading frame of interest. If the replaced gene is essential for cell viability, all transformed lines remained heteroplastomic even after repeated cycles of regeneration under highly selective pressure. This results in a balance of a fairly constant ratio between the wild-type and transformed genome copies. Using this strategy, the essential roles of chloroplast *ycf1*,

ycf2, *caseinolytic protease P1* (*clpP1*), and *accD* in cell viability have been revealed (Drescher *et al*., 2000; Shikanai *et al*., 2001; Kode *et al*., 2005). A further study on *clpP1* gene using the chloroplast transformation strategy in combination with the Cre-loxP system showed that the *clpP1* gene product is essential for the execution of the normal shoot developmental program in tobacco seedling (Kuroda and Maliga, 2003).

clpP1 is a proteolytic subunit of the ATP-dependent Clp protease, which is thought to be responsible for the majority of protein degradation in plastids (Kuroda and Maliga, 2003). Besides the plastid encoded CLPP1 subunit, the Clp protease complex contains four another proteolytic subunits (CLPP3-P6) and four non-proteolytic CLPR subunits (CLPR1-R4), all of which are encoded by the nuclear genes (Olinares et al., 2011). These proteolytic and non-proteolytic subunits constitute the tetradecameric Clp protease core, which consists of one heptameric R-ring with CLPP1, CLPR1, CLPR2, CLPR3, and CLPR4 in a 3:1:1:1:1 ratio, and one heptameric P-ring with CLPP3, CLPP4, CLPP5, and CLPP6 in a 1:2:3:1 ratio (Olinares et al., 2011).

Genetic studies on various *clpP* mutants in *Arabidopsis* showed the Clp protease system was essential for embryo development in *Arabidopsis*. Interestingly, the severity of the phenotypes for the various *clpPR* null mutants differs greatly (Sjögren *et al*., 2006; Kim *et al*., 2009, 2013; Koussevitzky *et al*., 2005; Zheng *et al*., 2006). Complete loss of CLPP4 or CLPP5 subunits arrested the embryo development at the globular stage, whereas complete loss of CLPR2 or CLPR4 subunits arrested embryogenesis at the cotyledon stage, and resulted in seedling lethal (Kim *et al*., 2009, 2013). The null mutants for *ClpP3* gene germinate, and resulted in seedling lethal as well (Kim *et al*., 2013). The downregulation lines for *ClpP6* and *ClpR1* genes can germinate and produce seed, but give rise to abnormal plants with reduced greening, reduced photosynthesis, and delayed development phenotypes (Sjögren *et al*., 2006; Kim *et al*., 2009). Since the lack of null mutants for *ClpP6* gene, it is still unknown

whether CLPP6 subunit plays an essential role in embryogenesis. Unlike other members in Clp protease core, there is a functional redundancy between CLPR3 and CLPR1 subunits, thus their roles in embryogenesis can only be determined in the double null mutant. Although *clpP1* was shown to be cell viability and shoot meristem development in tobacco, its role in embryogenesis is still unknown (Kuroda and Maliga, 2003). Together these data, the different phenotypes of null mutations in the subunits of the Clp protease core suggest different contribution of each subunit to the activity of Clp protease. Although Clp protease system is essential for embryogenesis in *Arabidopsis*, the underlying mechanism is still not clear, which may rely on identification of the direct substrates of Clp protease.

The plastid *accD* gene encodes the β-carboxyl transferase (β-CT) subunit of the plastid heteromeric acetyl-CoA carboxylase (He-ACCase) (Wakasugi *et al*., 2001). In plastids, there are two types of ACCases, homomeric one and heteromeric one (Ohlrogge and Browse, 1995). The homomeric ACCase (Ho-ACCase) is a multifunctional enzyme encoded by a single nuclear gene. By contrast, the He-ACCase is a multisubunits complex composed of four subunits, with biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and α-CT, encoded by three nuclear genes, and β-CT encoded by the plastid *accD* gene (Sasaki and Nagano, 2004). ACCase produces the malonyl-CoA which is used for *de novo* biosynthesis of fatty acid in plastids. After being synthesized, fatty acids are either used by plastids or transported into cytosol. Regarding the capacity for malonyl-CoA biosynthesis in plastids, plant species are divided into three groups (Schulte *et al*., 1997). Some species depend exclusively on the He-ACCase (grapevine type), some rely only on a Ho-ACC (corn type), and others use both enzymes (canola type, i.e. *Arabidopsis*). The genetic studies on the interaction between the plastid He- and Ho-ACCases show that the He-ACCase in *Arabidopsis* is the major form contributing to the biosynthesis of malonyl-CoA (Babiychuk *et al*., 2011). Although there is a functional redundancy between these two types of ACCases, the plants can tolerate the absence of the

Ho-ACCase, but the function of He-ACCase is essential for cell viability and its absence conditions the embryo lethal phenotype (Babiychuk *et al*., 2011). In addition to the plastid ACCases, there is also a cytosolic Ho-ACCase (Konishi and Sasaki, 1994). The malonyl-CoA produced by the cytosolic Ho-ACCase is used in various reactions: for example, the synthesis of flavonoids and anthocyanins, the synthesis of very long-chain fatty acids, the malonylation of D-amino acids, and malonylation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, which reduces the rate of ethylene production (Sasaki and Nagano, 2004). Malonyl-CoA cannot pass through the plastid envelop and must be synthesized separately in both plastid and cytosol according to cell requirements (Sasaki and Nagano, 2004; Babiychuk *et al*., 2011).

The functional studies on the subunits of plastid He-ACCase complex showed the BCCP subunit is essential for cell viability (Li *et al*., 2011). The loss of function in BCCP protein leads to a defect in the biosynthesis of fatty acid hence embryo lethality, which suggests the defect in fatty acid biosynthesis cause the abortion in embryogenesis in *Arabidopsis* (Li *et al*., 2011). The BC subunit is also required for the synthesis of fatty acid, and the antisense line conditions retarded plant growth phenotype in tobacco (Shintani *et al*., 1997). Since the lack of the null mutants in the BC and α-CT subunits, their roles in embryogenesis are still not clear.

The mechanism that fatty acids essential for embryogenesis may be underlying its roles as the building blocks of the membranes (Bloom *et al*., 1991). In plants, the major fatty acids have a chain length of 16 or 18 carbons and contain from one to three *cis* double bonds, and the five fatty acids (18:1,18:2,18:3,16:0, and in some species, 16:3) make up over 90% of the acyl chains of the structural glycerolipids of almost all plant membranes (Ohlrogge and Browse, 1995). The membranes not only physically divide all subcellular and cellular compartments, but also serve as the matrix for several important metabolic processes, e.g. photosynthesis and oxidative respiration (Bloom *et al*., 1991). In plastids, the inner and outer envelope membranes as well as thylakoid membranes are composed mainly by two types of galactolipids:

MGDG and digalactosyldiacylglycerol (DGDG), which have splayed polyunsaturated fatty acid tails and a head group with one and two galactoses, respectively (Block *et al*., 1983). The MGD1 enzyme in maize and Arabidopsis catalyzes the transfer of a galactosyl residue from UDP galactose to the *sn-3* position of diacylglycerol (DAG) to form MGDG (Benning and Ohta, 2005). As mentioned above, the maize MGD1 is encoded by the *O5* gene (Myers *et al*., 2011). The *o5* null mutant is defective in the production of MGDG and DGDG, reducing the level of thylakoid membranes, disrupting the granal organization, and causing arrest in both embryo and endosperm development (Myers *et al*., 2011). In *Arabidopsis*, the *mgd1* null mutant was embryo lethal as well, but germinated as small albinos in the presence of sucrose (Kobayashi *et al*., 2007). The germinated *mgd1* seedlings are short in both MDGD and DGDG, and had disrupted photosynthetic membranes, leading to the photoautotrophic growth (Kobayashi et al., 2007). These data show an essential role of galactolipids (i.e. MGDG and GDGD) in embryogenesis in both maize and *Arabidopsis*, and reveals the essential role of galactolipid synthesis, not only fatty acid synthesis in embryogenesis. Since the *mgd1* mutation affects the composition and structure of thylakoid membranes, it is anticipated that the photosystem imposed in the thylakoid membranes is essential for embryogenesis. However, the prior studies that examined a chlorophyll-deficient mutant (Frick et al., 2003) and *in vitro* embryo development (Sauer and Friml, 2004) have shown that photosynthesis is not required for embryogenesis. So, the defective embryo development observed in the *mgd1* null mutants in maize and *Arabidopsis* must be independent of the photosynthetic dysfunction caused by any impairment of thylakoid membranes. It is likely that perturbations in the plastid membranes could also affect membrane protein complexes that function in processes other than photosynthesis. For example, the biosynthesis of fatty acids and the plastid protein import machinery are both involved the integral membrane protein complexes (Joyard *et al*., 2010; Kessler and Schnelll, 2009). By this way, membrane composition may potentially affect multiple critical protein

complexes involved in plastid processes that are required for cellular function and plant viability, thus is essential for embryogenesis.

To summarize, the plastid genes (*clpP1*, *accD*, *ycf1*, and *ycf2*) are essential for cell viability in tobacco and the cellular process that *clpP1* and *accD* gene products taking part in (i.e. Clp protein degradation and the fatty acid biosynthesis pathways) are essential for embryogenesis in *Arabidopsis*. Thus, the essential role of plastid translation in embryogenesis may be due to the requirement of plastid translation machinery to express these important genes.

1.4 The unequal requirement of plastid translation for embryogenesis in maize and *Arabidopsis*

Although the plastid translation has been proven to be essential for embryogenesis in plants, there is evidence otherwise indicating that plastid translation is not equally important to embryogenesis between *Arabidopsis* and maize. For example, the nucleus encoded CRS2-associated factor 2 (CAF2) is required for group II intron splicing in chloroplast *rps12* gene (Asakura and Barkan, 2006; Ostheimer *et al*., 2003). Loss of function in CAF2 causes plastid ribosome deficiency in both maize and *Arabidopsis*, but in *Arabidopsis* it results in embryo lethality whereas in maize it allows normal embryogenesis and conditions an *albino* seedling phenotype. Similar results were reported with plastid PPR2, PPR4, PPR5 and THA8 (thylakoid assembly 8) proteins (Cushing *et al*., 2005; Khrouchtchova *et al*., 2012; Beick *et al*., 2008; Schmitz-Linneweber *et al*., 2006; Williams and Barkan, 2003; Lu *et al*., 2011). All of these proteins function in plastid RNA metabolism, however, null mutations in these genes cause plastid ribosome deficiency. Again, mutations of the orthologs in *Arabidopsis* cause embryo arrest and lethality, whereas in maize the loss of their functions allows normal embryogenesis and conditions *albino* seedlings.

When comparing the plastid genomes of maize and *Arabidopsis*, the *clpP1* gene is present in both of the two species (Sato *et al*., 1999; Maier *et al*., 1995). This challenges the essential role of *clpP1* in embryogenesis of maize. In addition, the

expression of plastid *clpP1* gene is inhibited in the maize *albino* mutants,, but embryo development proceeds normally. Consistently, Cahoon *et al*. (2003) reported that the *clpP1* gene in plastid genome of maize could be eliminated but didn't affect cell viability. Unlike *clpP1* gene, the plastid genes *accD*, *ycf1* and *ycf2* are found in the plastid genome of *Arabidopsis*, but missing from that of maize. In maize, the palstid *accD* gene is transferred to the nuclear genome, and the production of fatty acid in plastids is carried out by the plastid Ho-ACCase (Asakura and Barkan, 2006). That means the absence of plastid translation in maize doesn't affect the fatty acid synthesis, and there should be enough fatty acids produced by the nuclear-encoded Ho-ACCase for cell activities. Bryant *et al*. (2011) proposed the expression of *accD* gene in plastids is the requirement of plastid translation for embryogenesis in *Arabidopsis*, not in maize.

However, there are still two questions cannot be explained by the *accD* pathway. First, what is the mechanism that plastid translation regulating embryogenesis in maize since all of the known *emb* mutants in maize are defective in plastid translation? Second, there are two types of maize mutants: *emb* and *albino* mutants, defective in plastid translation. Embryogenesis is arrested in the *emb* mutants, but proceeds normally in the *albino* mutants, which suggests the plastid translation is essential for embryogenesis in the *emb* mutants, but not required in the *albino* mutants. What is the mechanism that makes the different phenotypic expression of the two types of plastid translation mutants in maize?

1.5 Functions of WHYIRLY family proteins in plants

The nuclear encoded WHIRLY1 (WHY1) proteins are known to have versatile roles. In barley and *Arabidopsis*, WHY1 is dual-localized in both the nucleus and the chloroplast (Desveaux *et al*., 2004; Grabowski *et al*., 2008; Krause *et al*., 2005). In the nucleus, it was shown to act as a transcription activator for pathogen related gene expression in *Arabidopsis* and potato (*Solanum tuberosum*) (Desveaux *et al*., 2004; Desveaux *et al*., 2000), and repressor for the kinesin-like protein 1 (KP1) in

Arabidopsis (Xiong *et al*. 2009). It was also implicated in modulating the homeostasis of telomere length in *Arabidopsis* (Yoo *et al*., 2007). In chloroplasts, WHY1 is implicated in the repair of plastid genome, and is necessary for the genome stability in maize and *Arabidopsis* (Cappadocia *et al*., 2010; Maréchal *et al*., 2009). In maize, WHY1 is also essential for the biogenesis of plastid ribosome. The severe loss of function allele *why1-1* in the standard genetic background and B73 conditions *albino* seedlings (Prikryl *et al*., 2008; Sosso *et al*., 2012).

1.6 Objectives of the project

According to the above review, plastid translation plays an essential role in plant embryogenesis. The mechanism that plastid translation regulating embryogenesis may be due to its essential role in the expression of several plastid encoded genes, i.e. *clpP1*, *accD*, *ycf1*, and *ycf2*, all of which are shown to be required for cell viability in tobacco. Moreover, the cellular processes that *accD* and *clpP1* taking parting in are essential for embryogenesis in *Arabidopsis*, thus indirectly demonstrates the requirement of *accD* and *clpP1* gene products for embryogenesis. How, there is still a confusion that plastid translation is essential for embryogenesis in maize *emb* mutants, but not in the maize *albino* mutants. In both of the *emb* and *albino* mutants, plastid translation is impaired. The goal of this study on the maize *embryo defective 16* (*emb16*) mutants is to further unravel the mechanism regulating embryogenesis in maize, and if possible, provide an answer to the above confusing question. And our objectives in the present study are to (1) characterize the *emb16* mutant phenotype, (2) cloning of the gene causing the *emb16* mutant phenotype, (3) study the functions of EMB16 protein.

Chapter 2 Methods and Materials

2.1 Plant materials

The mutants of *emb16*, *emb12-1*, and *emb14-1* were derived from the UniformMu transposon tagging population which is created by introgressing the *Mutator* (*Mu*) active line into a W22 inbred background (McCarty *et al*., 2005). All these mutations were maintained in a W22 genetic background. For developmental analyses and population generation, the plants were grown in the greenhouse of the Chinese University of Hong Kong and manually pollinated. The *iojap* (*ij*) seed stock and the inbred lines were provided by the Maize Genetics Cooperative Stock Center. The seeds of heterozygous *why1-1* and *why1-2* were kindly provided by Dr. Alice Barkan (Oregon State University).

2.2 Embryo rescue

Kernels from *emb16* segregating ears at 7, 10, 14, and 20 days after pollination (DAP) were left in a Na hypochlorite: dH2O (1:2) solution for 20 min and then washed by sterile water for three times (15-30 min/each time). The mutant embryos are distinguishable from the WT ones by their reduced size and abnormal structure. The mutant and WT sibling embryos were excised and separately transferred to petridishes containing basal MS medium (Sigma) supplemented with 3% sucrose and solidified with 0.3% Gelzan (Sigma). Embryo culture was maintained in a growth chamber at 25 ºC with a 16/8 h light/dark photoperiod. After 15 days of culture, the germination percentage was determined.

2.3 Histological analysis of the *emb16* seed development

Developing WT and mutant kernels were harvested from the self-pollinated segregating ears at 5, 6, 7, 10, 14, 21, and 27 DAP. The kernels were cut along longitudinally into three parts. The central slice containing the embryo was fixed in 4% (w/v) paraformaldehyde for overnight at 4 ºC (Sylvester and Ruzin, 1994). The

fixed material was dehydrated in a graded ethanol series, infiltrated and embedded in paraffin, sectioned at 8 to 10 μm with microtome (Jung Biocut 2035, Leica, Germany), and mounted on slides as described (Sylvester and Ruzin, 1994). The sections were then deparaffinized, stained with safranin O and fast green (Johansen, 1940), and observed with microscope (Eclipse E80i, Nikon, Japan).

2.4 Transmission electron microscopy analysis

The *emb16* and WT embryos were dissected from a segregating ear at 6, 7, 8, and 14 DAP using stereomicroscope. At 6 DAP, the *emb16* embryo was distinguished from the WT by the size and structure of the embryo proper, and confirmed by PCR genotyping on the endosperm tissue. For chloroplast structurally analysis, sections 1 cm below the tip of the second leaf from two-leaf stage seedlings were collected and cut into small pieces. The fixation, dehydration, infiltration, and embedding of embryo and leaf tissues were performed as described (Spurr, 1969). Ultrathin sections of 70 nm were cut using diamond knife (Diatome, Electron Microscopy Sciences, USA) and ultramicrotome (Ultracut S, Leica, Germany), and lifted onto 2 mm copper grids. Grids were stained in uranyl acetate and lead citrate prior to observe with transmission electron microscope (H-7650, Hitachi, Japan).

2.5 DNA extraction and Southern blot analysis

Maize genomic DNA was extracted from two-leaf stage seedlings using the urea extraction method (Chen and Dellaporta 1994). 1g fresh weight of leaf tissues was ground in liquid nitrogen and extracted with 5 ml of DNA extraction buffer (7 M urea, 0.3 M NaCl, 50 mM Tris-HCl, 24 mM EDTA, and 1% sarkosine, pH 8.0). After mixing with 4 ml phenol-chloroform-isoamyl alcohol (25:24:1), the extraction was carried out with gentle shaking for 30 min at room temperature. The mixture was separated by centrifugation at 4800 g for 15 min. The aqueous phase was transferred to a new tube and mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 3.8 ml isopropanol. DNA was pelleted at 4800 g for 5 min, washed with 70% ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

About 10 μg genomic DNA was digested with *BamHI*, *HindIII*, or *EcoRI* (New England Biolabs) at 37 ºC for 6 hr. The DNA was resolved on 0.7% (w/v) agarose gel, denatured, and transferred to nylon membranes (Amersham, UK). The membrane was cross-linked and hybridized. The probe was labeled by [α-^{32}P]dCTP (3,000 Ci mol^{-3}) using the DNA labeling beads (Ready-To-Go, GE Healthcare, USA), and purified with ProbeQuant G-50 micro column (GE Healthcare, USA).

For probes used in Southern analyses, the *Mu1*/*Mu2* probe was derived from the *HinfI* fragment of the *Mu1* element, which contains the internal sequence without the terminal inverted repeat (TIR) region. For *Mu8* probe, a DNA fragment internal to the *Mu8* TIRs was used (Walsh *et al*., 1998).

2.6 Cloning of *Mu*-flanking sequence by TAIL-PCR

The improved high efficiency TAIL-PCR method (Liu and Chen 2007) was adapted for amplification of the *Mu*-flanking sequence that were identified in the co-segregation analysis. *EcoRI* digested genomic DNA (*emb16*/+) was resolved on 0.7% (w/v) agarose gel. DNA fragments with size from 3.0 to 3.5 kb for the *Mu8*-flanking fragment, and from 7.3 to 7.7 kb for the *Mu1/Mu2*-flanking fragment were recovered and used as PCR templates. *Mu8* and *Mu1/ Mu2* specific nested primers were designed based on the internal sequences of the Mu8 or *Mu1* and *Mu2*, with Mu8-F1, Mu8-F2, Mu2-F1 and Mu2-F2 for the forward direction and Mu8-R1, Mu8-R2, Mu2-R1 and Mu2-R2 for the opposite direction (Table 2.1). Four arbitrary degenerated primers (AD3-1 to AD3-4) were designed partially based on the report by Liu and Chen (2007). The 5' embedded primer in these four AD primers (i.e. AD3 primer) was derived from a non-maize sequence (Table 2.1) and a BLAST analysis against the maize genomic sequence in the GenBank did not identify any priming site. Three rounds of TAIL-PCR were carried out. In the first round, *Mu8* or *Mu2* primers in combination with AD3-1, AD3-2, AD3-3, or AD3-4 were used. The second and third rounds used the nested *Mu8* or *Mu2* or TIR primers (TIR6 or TIR8) in combination with AD3 (Table 2.1). The TIR8 primer is a mixture of the TIR8.1, TIR8.2, TIR8.3 and TIR8.4 primers in a 2:4:4:1 ratio (Settles et al., 2004). The PCR conditions were

Table 2.1 Primers used in this paper.

Name	Sequence (5'-3')	Reference
TIR1	TTAGTCTCCTGTCCCCGTTTACCGT	This paper
TIR8.1	CGCCTCCATTTCGTCGAATCCCCTS	Settles *et al*., 2004
TIR8.2	CGCCTCCATTTCGTCGAATCCSCTT	Settles *et al*., 2004
TIR8.3	SGCCTCCATTTCGTCGAATCCCKT	Settles *et al*., 2004
TIR8.4	CGCCTCCATTTCGTCGAATCACCTC	Settles *et al*., 2004
TIR6	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC	Settles *et al*., 2004
AD3-1	AGTTTTTGGGTGGTGG(G/C/A)N(G/C/A)NNNGGAA	Liu and Chen, 2007; this paper
AD3-2	AGTTTTTGGGTGGTGG(G/C/T)N(G/C/T)NNNGGTT	Liu and Chen, 2007; this paper
AD3-3	AGTTTTTGGGTGGTGG(G/C/A)(G/C/A)N(G/C/A)NNNCCAA	Liu and Chen, 2007; this paper
AD3-4	AGTTTTTGGGTGGTGG(G/C/T)(G/A/T)N(G/C/T)NNNCGGT	Liu and Chen, 2007; this paper
AD3	AGTTTTTGGGTGGTGG	This paper
Mu1-F1	GACGAGCAACGGACGCACCTTCC	This paper
Mu2-F1	GTGAAGCCCTGCTCCCGCACCTACT	This paper
Mu2-F2	GACGGAGATGCGACGGAGAAAAAGGGT	This paper
Mu2-R1	GGCCGCGGCGAGGTGGAGC	This paper
Mu2-R2	GCGCGGCGACGGAGACAAGAAGAGT	This paper
Emb16-F1	ACAGAGCAGGACAGGATAGACC	This paper
Emb16-F2	CCGCCTGTCTCCTCCTCG	This paper
Emb16-R1	CCTTCGCCATTCGTACTCAGAC	This paper
Emb16-R2	TGAAAGGGTCATGAAAGAACTCG	This paper
Mu8-F1	AGTCGGAGGTGGAGAAGTCGGAGAGGC	This paper
Mu8-F2	AGTCGGAGGTGGAGAAGTCGGAGAGGC	This paper
Mu8-R1	GGGGACCGCATCTCCGTCATCGT	This paper
Mu8-R2	AGGCGAACGGTAAACGGGGACAGG	This paper

according the report, with minor adjustment of temperatures depending on the primers and DNA polymerase. In most cases, several fragments were amplified after the second or third round of TAIL-PCR. These fragments were recovered and ligated into vector (pCR2.1-TOPO, Invitrogen, USA), and sequenced.

2.7 Fractionation of chloroplasts and nuclei from maize leaves

The fractionation of chloroplasts was based on the procedure as previously reported with modifications (Cline 1996). About 25 g leaves of two-leaf stage W22 seedlings were chopped and ground in a blender in ice cold 250 ml grinding buffer [GR buffer; 50 mM HEPES-KOH pH7.5, 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM Na-ascorbate and 1% (w/v) bovine serum albumin]. The grinding was conducted in four 10 second pulses at low speed setting. The ground was filtered through one layer of pre-wet Miracloth (Calbiochem, USA). The solution was centrifuged at 3,000 g for 8 min at 4°C. The pellet, which contains chloroplasts, was resuspended in 5 ml ice-cold GR buffer and laid on top of a prepared Percoll gradient (50% 2× GR buffer and 50% Percoll), and centrifuged at 6,500 g for 15 min. The top band was discarded. The lower band was collected and diluted 3 folds with import buffer (50 mM HEPES-KOH pH8.0, 330 mM sorbitol). The chloroplasts were centrifuged down at 2,600 g for 8 min, then washed by resuspending in 15 ml import buffer and centrifuged at 2,600 g for 4 min. The chloroplasts were suspended in an appropriate volume of import buffer to get about 1 mg chlorophyll per ml.

The isolation of intact nuclei from maize leaves was conducted as previously reported (Zhang *et al*., 1995). The integrity and purity of the nuclei were checked by microscopic observation.

2.8 RNA extraction and RT-PCR analysis

For RNA isolation, the seedlings homozygous for *why1-1*, *why1-2*, or *why1-3* were genotyped by PCR using primers of Emb16-R2 in combination with Mu2-F1 for *why1-3*, and TIR8 for *why1-1* and *why1-2* (Table 2.1). Leaf RNA was extracted from the middle of the second leaf with RNeasy mini kit (Qiagen, Germany). For RT-PCR,

the first-strand cDNA was synthesized using the PrimeScript® reverse transcriptase kit (Takara, Japan). Emb16-R1 in combination with Emb16-F2 or Emb16-F1, which cross the *Mu* insertion site were used to amplify the *why1* transcripts in *why1-1* and *why1-3* albino leaves (Table 2.1).

2.9 Protein extraction and Western blotting analysis

Proteins from maize leaves of WT, *why1-1*, *why1-2* and *why1-3* were prepared as previously described (Ding *et al*., 2011), and the concentration was quantified by protein assay kit (Bio-Rad, USA). Ten microgram protein of each sample was subjected to Western blot as described (Kesari *et al*., 2012).

Chapter 3 Results

3.1 Phenotypic characterization of *emb16* mutant

The *emb16* mutant is monogenetic, recessive (288:91, WT:*emb*, 3:1, X^2=0.2 p>0.50) and homozygous lethal. In maturity, the mutant kernel is about the WT size and often with dark pigmentation, a feature that is observed in kernels of many maize *emb* mutants (Figure 3.1). The endosperm appears to be normal and is filled with starch. However, the embryo is defective. At 20 DAP, the mutant embryo is arrested at transition stage, only develops a pre-embryo proper and a suspensor. The WT kernel at the same stage develops an embryo with primary shoot, root and scutellum. Due to embryo lethality as homozygotes, the *emb16* mutant was propagated as heterozygote. We attempted to rescue the mutant embryo by culturing it on the MS medium at 7, 10, 14, and 20 DAP, but all attempts were not successful. This is in contrast with the embryo rescue on another three *emb* mutants, i.e. *emb*-7191*, *emb*-7182*, *emb*-7192*, whose embryogenesis was arrested at or before the transition stage (Consonni *et al*., 2003).

To determine the impact of *emb16* mutation on embryogenesis, we compared the embryo development process between the *emb16* mutant and the WT. For a precise comparison, we analyzed the mutant and its WT siblings on a segregating ear since these seeds developed at identical conditions. The mutant embryos were identifiable from the WT at as early as 5 DAP by the smaller size and structure of the underdeveloped embryo (Figure 3.2, A and H). Under our growth condition, the WT embryo establishes the apical-basal axis at 5 DAP and forms primary scutellum, coleoptile and shoot and root primordia at 10 DAP (Figure 3.2, A-D). By 14 DAP, the embryo develops all the structures of a mature embryo, but at about half the size (Figure 3.2E). By 21 DAP, the embryo has developed primary root and 3 to 4 primary leaves (Figure 3.2F). At 27 DAP, the embryo enlarges and forms 4 to 6 primary leaves (Figure 3.2G). In contrast, *emb16* embryo establishes the apical-basal axis at 6 DAP,

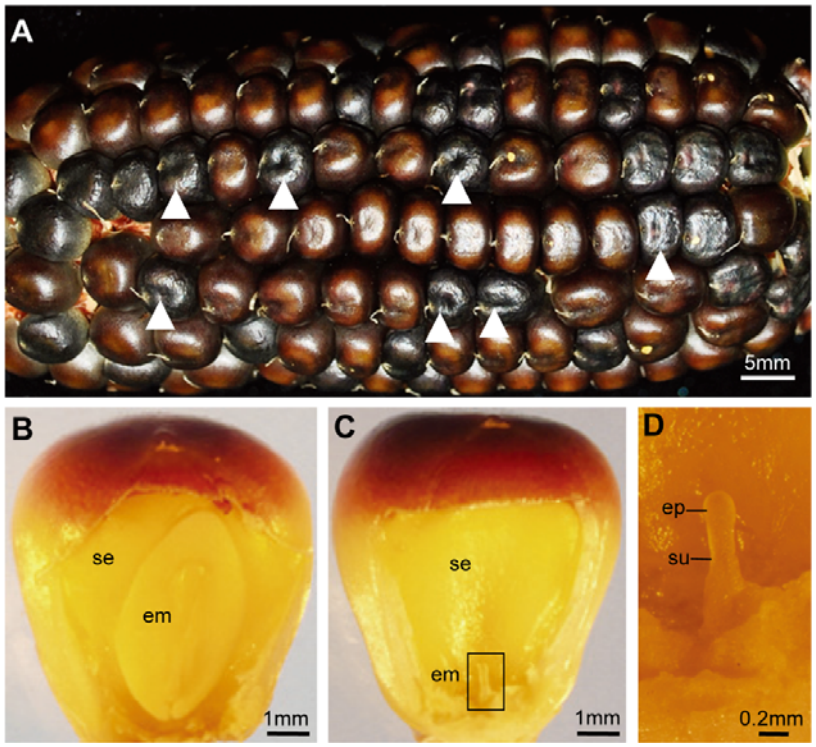


Figure 3.1 Phenotype of *emb16* mutant in W22 background.

(A) An ear segregating the *emb16* mutant. Arrows point to the *emb16* mutant kernels. (B) Mature WT kernel. (C) Mature *emb16* mutant kernel. (D) Enlarged view of the mutant embryo in (C). se: starchy endosperm; em: embryo; ep: embryo proper; su: suspensor. Scale bars as indicated.

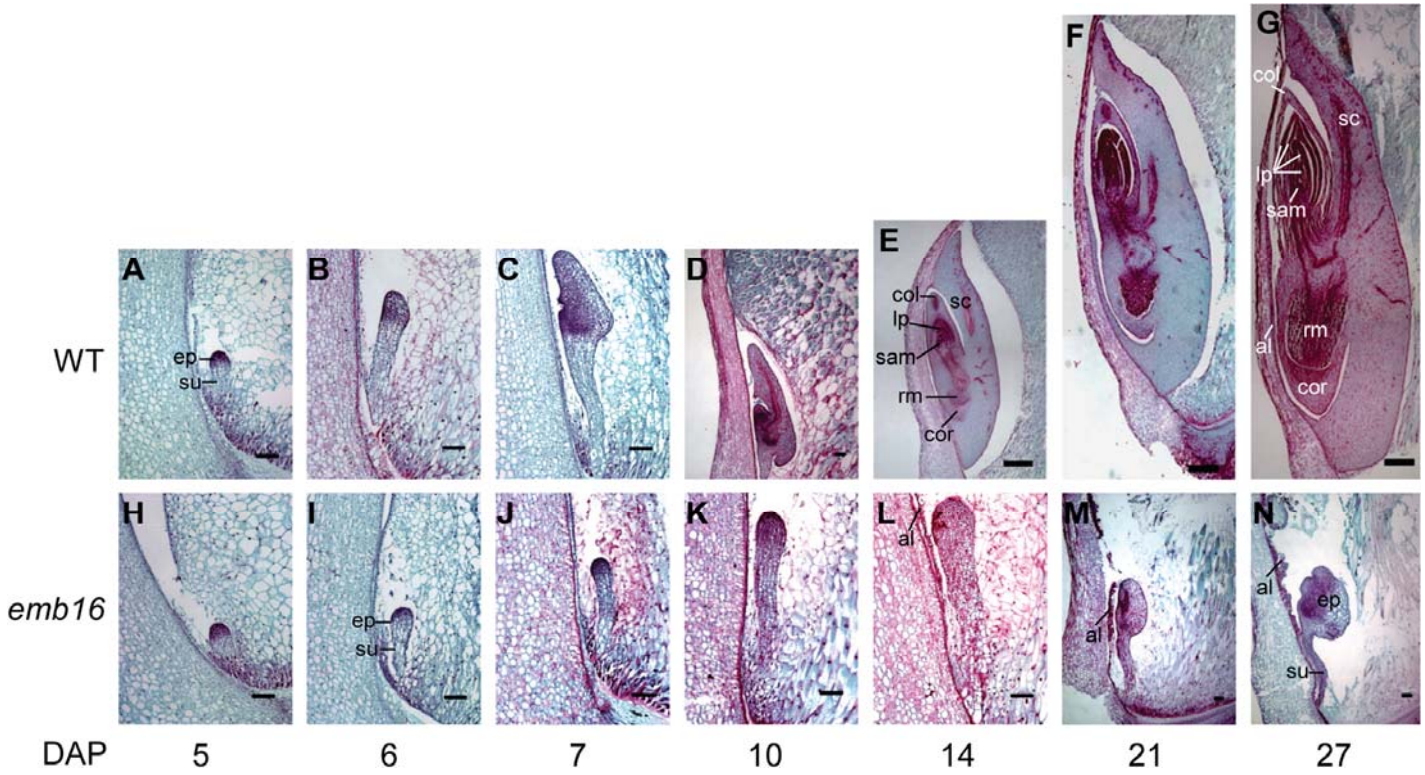


Figure 3.2 Embryo development in *emb16* mutant.

The WT and *emb16* mutant kernels from segregating ears were sectioned from 5 to 27 DAP. Longitudinal sections of maize kernels were stained with Safranin O and fast green. At 5 and 6 DAP, the *emb16* embryos were distinguished from the WT by the size and structure of embryo proper. al: aleurone cell; col: coleoptile; cor: coleorhiza; ep: embryo proper; lp: leaf primordia; rm: root meristem; sam: shoot apical meristem; sc: scutellum; su: suspensor. Scale bars: E, F, and G =1 mm; others =0.1 mm.

and appears to differentiate until 10 DAP with the increased cell density at the adgerminal face of the embryo proper (Figure 3.2, I-K). At 14 DAP, the mutant embryo ceases differentiation and shows signs of degeneration (Figure 3.2L). At 21 and 27 DAP, the embryo forms a tumor like head structure which is still attached to a suspensor (Figure 3.2, M and N). This developmental analysis indicates that the *emb16* mutation arrests the embryo development at the transition stage as previously defined by Abbe and Stein (1954), and the mutant embryo could not differentiate to form any organ of a WT embryo.

Compared with the severe defects in embryo development, the development of *emb16* endosperm is almost normal (Figure 3.3). At 5 DAP, the kernel was still not filled with the endosperm tissue, and the size of *emb16* endosperm was similar with that of WT (Figure 3.3, A and D). By this stage, the endosperm in both WT and mutant has been specified to form the four endosperm domains, i.e. aleurone cell layer, starchy endosperm, embryo-surround region, and basal transfer cell layer. From 5 DAP to 26 DAP, no striking difference in the size and structure of the four endosperm domains was observed between the mutant and WT, except the adgerminal face aleurone cells (Figure 3.3, B, C, E and F; Figure 3.4). At 14 DAP, the aleurone cells in the adgerminal face of the *emb16* kernel divides inwards (Figure 3.2L; Figure 3.4D). As the kernel develops, the aleurone cells continue the inward division, causing disorganization of aleurone cell layer (Figure 3.2, M and N; Figure 3.4, E and F). This defect in aleurone cell development is consistent with the dark pigmentation in the mutant kernel which is likely from the aleurone cells. From these observations, we conclude that (1) the *emb16* mutation also affects the endosperm cell development, but only at a lesser extent; (2) *emb16* is a typical *emb* mutant, specifically arresting embryo development at the transition stage, but allowing endosperm development almost normal.

3.2 Cloning of *Mu*-flanking fragments co-segregating with *emb16* mutant

The *emb16* mutant was isolated from the UniformMu population, which was created by introgressing the *Mu* active line into the W22 inbred background (McCarty

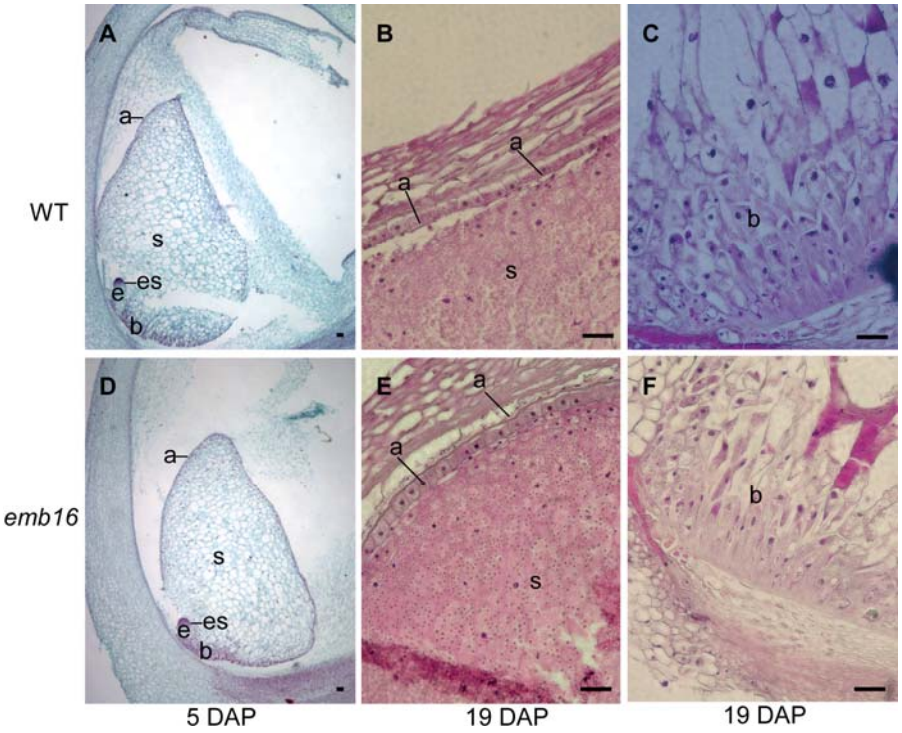


Figure 3.3 Endosperm development in *emb16* mutant.

(A) and (C): At 5 DAP, the endosperm tissue doesn't filled the kernel. The *emb16* endosperm size is simililar with that of WT, but the embryo size is smaller. By this stage, the endosperm has been specified to the four endosperm domains, i.e. aleurone cell layer, starchy endosperm, embryo surround region, and basal transfer cell layer. (B), (C), (E) and (F): At 19 DAP, no size and structural differences in the alerone cell layer, starchy endosperm, and basal transfer cells were observed between WT and *emb16* mutant. a: aleurone cells; b: basal transfer cells; e: embryo; es: embryo surround region; s: starchy endosperm. Scale bars =0.1 mm.

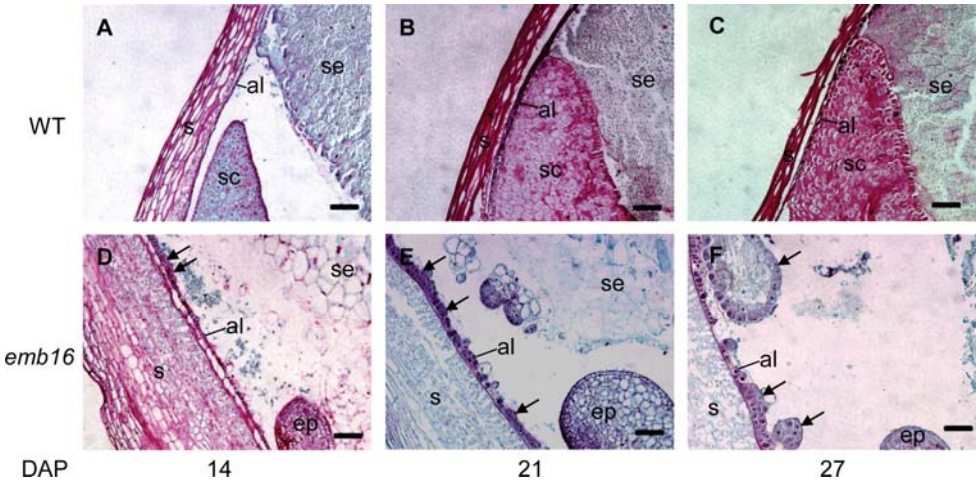


Figure 3.4 Abnormal aleurone cell development in the kernel germinal face of *emb16* mutant.

The WT and *emb16* mutant kernels from a segregating ear were sectioned from 14 to 27 DAP. Arrows point to abnormally dividing aleurone cells in *emb5* mutant. al: aleurone cells; s: seed coat; sc: scutellum in WT kernel; se: starchy endosperm; ep: embryo proper in *emb5* kernel. Scale bars = 0.1mm.

et al., 2005). The active *Mu* line was from Donald Robertson's collection and had been backcrossed to W22 for six times when the *emb16* mutation was isolated. After the isolation, the mutant was backcrossed two times to W22 again in an effort to reduce the active *Mu* copy number. As a result, the *emb16* mutation used in this study is in a nearly isogenic W22 background (99.6%).

Since the *emb16* mutation was created by utilizing *Mu*s as the mutagen, co-segregation analysis by Southern blot was performed with the purpose to tag the mutation with a *Mu*. To do this, *Mu* elements derived from the known active *Mu*s, including *Mu1*, *Mu2*, *Mu3*, *Mu8* and *MuDR* were used as probes. Since *Mu1* and *Mu2* share a high degree of sequence identity, a probe, i.e. *Mu1*/*Mu2*, derived from the ~1 kb *Hin*fI fragment of the *Mu1* element internal sequence, recognizes both the *Mu*s. The genomic DNAs from *emb16* segregating (+/-) and non-segregating (+/+) lines were digested using different restriction enzymes, including *HindIII*, *EcoRI*, and *BamHI*. This makes easier the seperation of the potential co-segregating *Mu*-flanking fragments on gel electrophoresis. When digested by *EcoRI*, one 3.2 kb fragment flanked by *Mu8* and one 7.5 kb fragment flanked by *Mu1* or *Mu2* were identified (Figure 3.5A; Figure 3.6A). When the population size increased to 40 individuals, it showed the same linkage, suggesting both *Mu* insertions were tightly linked with the *emb16* phenotype.

To reveal the *Mu* flanking sequences, we improved the previous *Mu*TAIL-PCR (Settles *et al*., 2004) in three aspects. First, we adapted it with an improved TAIL-PCR protocol (Liu and Chen, 2007) which increases the efficiency while reduces the reaction number. Second, we used size selected genomic DNA enriching the 3.2 or 7.5 kb *EcoRI* fragments as the PCR template. Third, we employed the *Mu1*/*Mu2* or *Mu8* specific primers coupled with arbitrary degenerate primers (referring to the Materials and Methods). The improved protocol was proven robust in extracting the *Mu*-flanking fragments, saving efforts in comparison to screening a genomic library (Walsh *et al*., 1998) and more efficient than the inverse PCR strategy

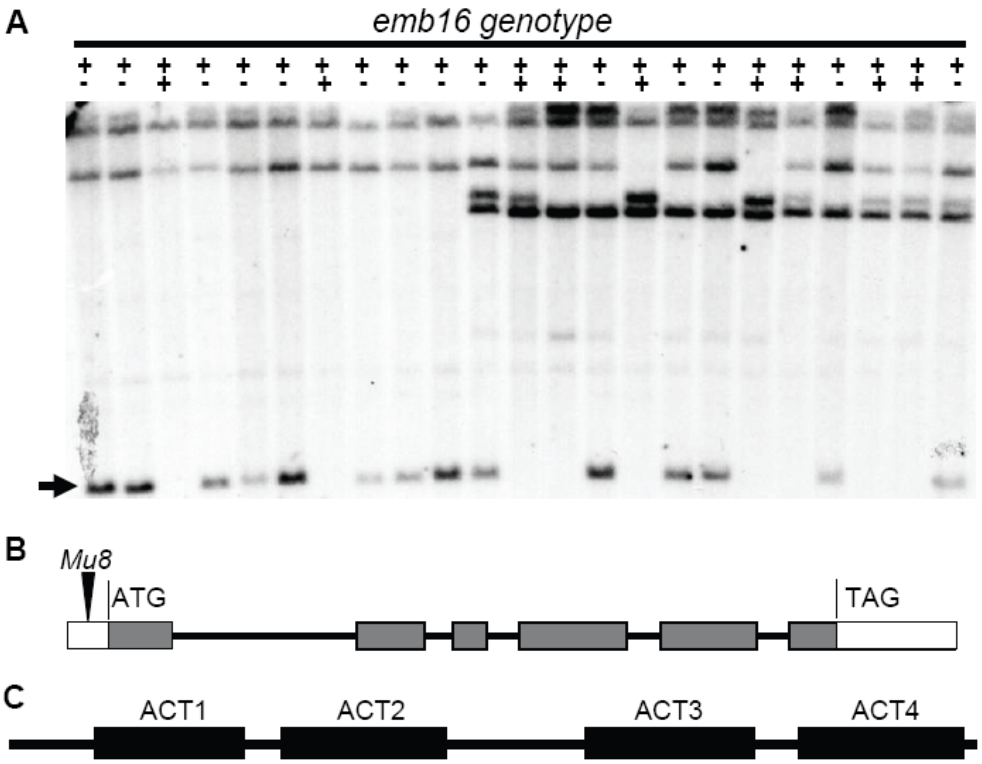


Figure 3.5 Cloning of Zm*ACR1* gene.

(A) Southern analysis of individual plants from an *emb16* segregating family by using a *Mu*8 specific probe. "++", WT and "+-", heterozygote for the *emb16* mutation. The genomic DNAs were digested with *EcoRI*. Arrow points to a 3.2 kb fragment that co-segregates with the *emb16* mutation. (B) Gene structure of Zm*ACR1* gene and the *Mu8* insertion site. Zm*ACR1* gene consists of 5 introns (lines) and 6 exons (filled boxes). Triangle is a *Mu8* insertion 69 bps unstream of translation start site. ATG: translational start site; TAG: translationanl stop site. C. Schematic diagram of ZmACR1 protein. It contains four ACT domains. The black boxes indicate the ACT domains.

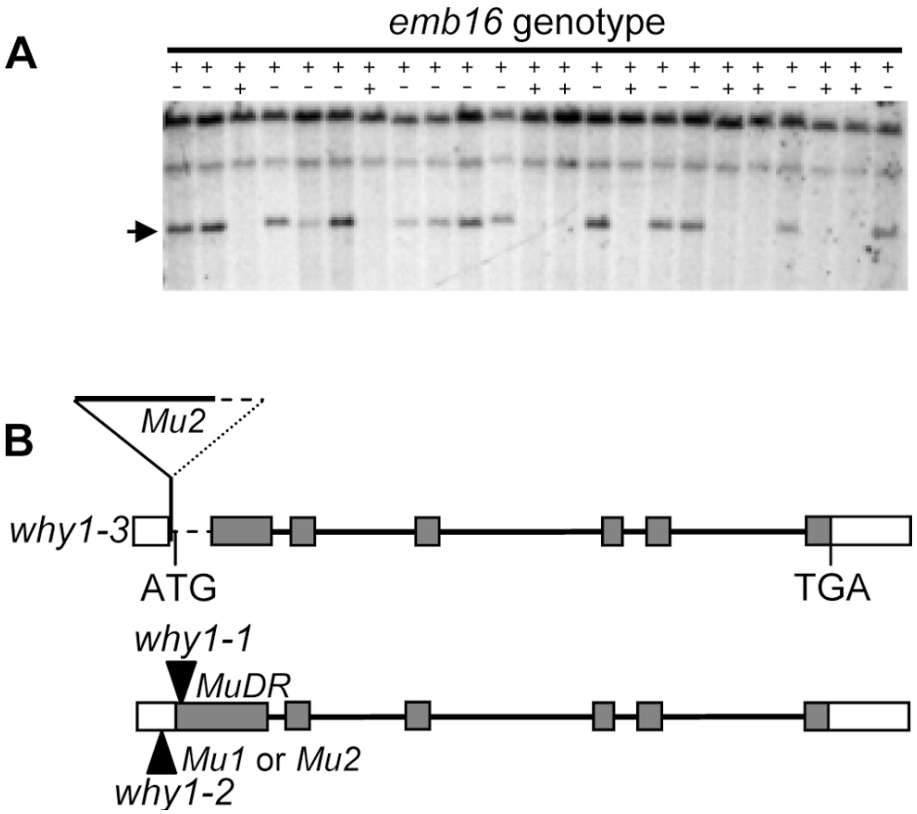


Figure 3.6 Cloning of *ZmWhy1* gene.

(A) Southern analysis of individual plants from an *emb16* segregating family by using a *Mu1*/*Mu2* probe. "++", WT and "+-", heterozygote for the *emb16* mutation. The genomic DNAs were digested with *EcoRI*. Arrow points to a 7.5 kb fragment that co-segregates with the *emb16* mutation. (B) Gene structure of *Why1* gene and *Mu2* insertion site in the *why1-3* allele. A fragment covering 380 bps *Mu2* and 245 bps *Why1* (dotted line) was deleted in the *why1-3* allele. Exons are boxes and introns are lines. Translated regions are filled boxes. Triangles are *Mu* insertions in the *why1-1* and *why1-2* allele (Prikryl *et al*., 2008).ATG: translation start codon, TGA: translation stop codon.

(Liu *et al*., 2013). After amplification, the PCR products were cloned and sequenced. The sequencing and BLAST results indicated that the 3.2 kb fragment corresponds to a *Mu8* insertion in the gene encoding ACT domain repeat protein 1 (*ACR1*; acc: EU963104; Figure 3.5B), and the 7.5 kb fragment corresponds to a *Mu2* insertion in the *Why1* gene (acc: EU595664; Figure 3.6B).

The *ACR1* gene contains six exons and the *Mu8* element is inserted the 5'-UTR, 69 bps upstream of the translation start codon (Figure 3.5B). The mazie ACR1 protein contains four ACT domains (Figure 3.5C), which was named after bacterial aspartate kinase, chorismate mutase and TyrA (prephenate dehydrogenase) (Aravind and Koonin, 1999). In bacteria, the ACT domain serves as amino acid-binding sites in some amino acid metabolic enzymes and transcriptional regulators, such as PII-uridylyl transferase/uridylyl-removing enzyme, 3-phosphoglycerate dehydrogenase, and Tyr and phenol metabolism operon regulator (Arcondéguy et al., 2001; Schuller et al., 1995; Pittard et al., 2005). In these proteins, the ACT domains are involved in the binding of amino acids, thus regulate the enzyme catalytic activities or transduce the signals. The ACR family is a novel type of ACT domain-containing protein family (Hsieh and Goodman, 2002). In *Arabidopsis* and rice, the ACR proteins contain two, three, or four ACT domains (Hsieh and Goodman, 2002; Liu, 2006; Sung *et al*., 2011). Besides the ACT domains, they have no homology to any known enzymes or protein motif in the PSI-BLAST conserved domain search (Hsieh and Goodman, 2002). This is in contrast to other ACT domain-containing proteins where the regulatory ACT domain is usually linked to an enzyme (Aravind and Koonin, 1999). Since the lack of *acr* mutants and the short of information on ACR protein functions, the role of ACR family in seed development is still not clear.

The *Why1* gene also contains six exons and the *Mu2* element is inserted in the first exon (Figure 3.6). In this mutation, the *Mu2* insertion caused a deletion of 380 bps in the *Mu2* element and 245 bps in the *Why1* first exon. The deletion includes the translation start codon of the *Why1* gene, suggesting that this allele may be null. The

Why1 gene has been reported in an *albino* seedling mutant (Prikryl *et al*., 2008). The strong allele *why1-1* carrying a *MuDR* insertion 35 bps downstream of the *Why1* translation start codon conditions ivory seedlings; the weak allele *why1-2* carrying a *Mu1*/*Mu2* insertion in the 5'-UTR conditions pale green seedlings. Since the suspected severe allele of *why1-1* is capable to develop viable seeds (normal embryogenesis), WHY1 protein is indicated to be not required for embryogenesis in maize.

Based on the above analyses, the *ACR1* gene shows a bigger possibility to be the causative gene for *emb16* mutant than the *Why1* gene.

3.3 Localization of ACR1 and WHY1 proteins

The previous studies showed an important role of plastids in embryogenesis (Tzafrir *et al*., 2004; Hsu *et al*., 2010; Bryant *et al*., 2011). So, we focused on the localization of ACR1 and WHY1 proteins in the beginning, especially the ACR1. If *ACR1* is the causative gene, it has a big possibility to be targeted into chloroplasts.

In the ACR protein family, the rice ACR7 and ACR9 proteins were localized in the nucleus (Hayakawa *et al*., 2006; Kudo *et al*., 2008), and the *Arabidopsis* ACR11 and ACR12 proteins were localized in the chloroplast (Sung *et al*., 2011). Analyses of the maize ACR1 protein sequences by both ChloroP (Emanuelsson *et al*., 1999) and Predotar (Small *et al*., 2004) algorithms didn't predict the presence of a chloroplast transit peptide, but by Nuc-Ploc predicted a nucleus localization sequence (Shen and Chou, 2007). To experimentally study the localization, the maize ACR1 was fused at the C-terminus with the GFP protein in a binary vector pGWB5 (Nakagawa *et al*., 2007). The fusion protein was transiently expressed in tobacco epidermal cells via Agrobacterium EHA105 infiltration. The fusion protein generated fluorescence in both nucleus and cytosol, but not in chloroplast (Figure 3.7A). This result suggests ACR1 protein was localized into both nucleus and cytosol, but didn't go to chloroplast. Since both nucleus- and cytosol-localized proteins could be essential for embryogenesis as well, e.g. cytosolic ribosomal protein S5, and DCAF1 (nuclear

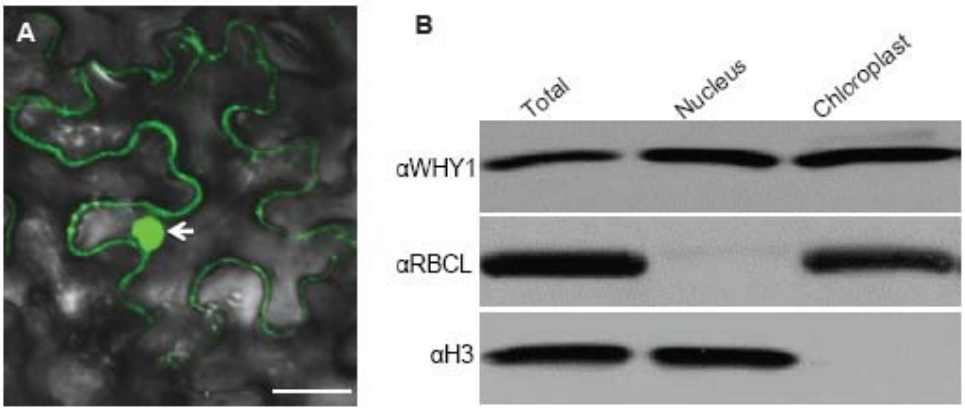


Figure 3.7 Introcellular localization of ACR1 and WHY1 in maize. (A) Confocal fluorescence microscopic detection of transiently expressed ACR1-GFP fusion protein in tobacco epidermal cells. The arrow points to the nucleus. Scale bar: 10 um; (B) Immunoblots of extracts from leaf and subcellular fractions: nucleus and chloroplast. The same blot was probed to detect a marker for nucleus (Histone H3; Owto Biotech. INC., China) and chloroplast (RBCL; Beijing Genomics Institute, China).

ubquitin E3 ligase) (SeedGenes), we still cannot rule out the possibility that *ACR1* is the causative gene.

The WHY1 protein was localized in chloroplast, in which it is essential for the biogenesis of plastid ribosome (Prikryl *et al*., 2008). However, orthologous WHY1 proteins in *Arabidopsis* and barley were shown to be dual-localized in the chloroplast and the nucleus (Grabowski *et al*., 2009; Krause *et al*., 2005). To test the nuclear localization of maize WHY1, immunodetection was performed. The nuclei and chloroplasts were purified from W22 seedling leaves and the proteins were extracted. The protein blot was hybridized by WHY1 antibody (generously provided by Dr. Alice Barkan). Cross-contamination was monitored by the chloroplast marker RUBISCO large subunit (RBCL) and the nuclear marker histone 3 (H3). As shown in Figure 3.7B, the WHY1 antibody recognized a single 27 kD protein in both the nuclear and chloroplast fractions. The size of WHY1 in the chloroplast is close to that in the nucleus, suggesting that WHY1 may have either a short or no transit peptide. No cross contamination was detected between the two fractions. This result confirms the chloroplast localization, and indicates that maize WHY1, similar to its orthologs in *Arabidopsis* and barley, is also localized in the nucleus.

From the localization results, we are still not sure which one is the potential causative gene for *emb16* mutant.

3.4 Separation of *acr1* and *why1* mutations

In addition to the localization studies, we separated the two linked mutations. By genotyping 253 seedlings from an *emb16* segregating ear (the total number of *emb* kernels from the same ear is 81, which were not genotyped), 8 individuals containing *why1* or *acr1* single mutation were screened out. The recombination frequency between the two mutations suggests that the genetic distance between *ACR1* and *Why1* genes is about 2.4 centimorgan. In the selfed progenies, *emb* kernels were exclusively obtained from the *why1* single mutants. By contrast, the selfed progenies from plants containing single *acr1* mutation didn't show any defect in seed development. They grew well, and produced seeds normally (data not shown). These

results indicated the *why1* mutation was more tightly linked with *emb16* phenotype, thus we shifted our attention to the studies on *Why1* gene.

3.5 Allelism test for *why1* mutation

To test whether the *Why1* is the causative gene, we obtained the previous *why1-1* and *why1-2* alleles from Dr. Alice Barkan (Prikryl *et al*., 2008). We crossed *emb16* heterozygotes with the *why1-1* and *why1-2* heterozygotes, respectively. The F1 of the *emb16*/+ × *why1-1*/+ crosses segregated mostly ivory seedlings, but also *emb* mutants (Figure 3.8). The sum of (*emb*+*albino*) accounted for ~25% of the total kernels, indicating a single recessive mutation. The crosses of *emb16*/+ × *why1-2*/+ segregated only pale green seedlings (Figure 3.8A). The ivory and pale green seedlings in all the crosses were genotyped and confirmed to be compound heterozygotes, *i.e. emb16:why1-1* or *emb16:why1-2*. Because these crosses produced *albino* seedlings and/or *emb* kernels, this result confirms that the mutation in the *Why1* gene is the cause of the *emb16* phenotype. We therefore named the *emb16* mutant *why1-3*.

3.6 The *why1-3* phenotypic expression is dependent on genetic backgrounds

The different phenotypic expression of *why1* mutation invokes two explanations, 1) *why1-3* is a null allele, whereas *why1-1* is leaky; 2) the genetic backgrounds determine the phenotypic expression of *why1* mutation. The first possibility assumes that a low level of WHY1 is sufficient for embryogenesis. To examine the WHY1 protein expression levels, we performed Western blot analysis on seedling leaves homozygous for *why1-1*, *why1-2* or *why1-3*. The WHY1 antibody detected a single band with expected size of WHY1 (~26 kD) in both WT and *why1-2*, but not in *why1-1* and *why1-3*, suggesting that *why1-2* is a leaky allele and both *why1-1* and *why1-3* are likely null alleles (Figure 3.9). The leaky nature in *why1-2* is consistent with the *Mu* insertion 38 bps upstream of the translation start codon which may allow leaky expression (Prikryl *et al*., 2008).

To analyze the *why1* transcripts in these alleles, RT-PCR was performed. In *why1-2* pale green leaves, a weak band with the size similar to WT was amplified

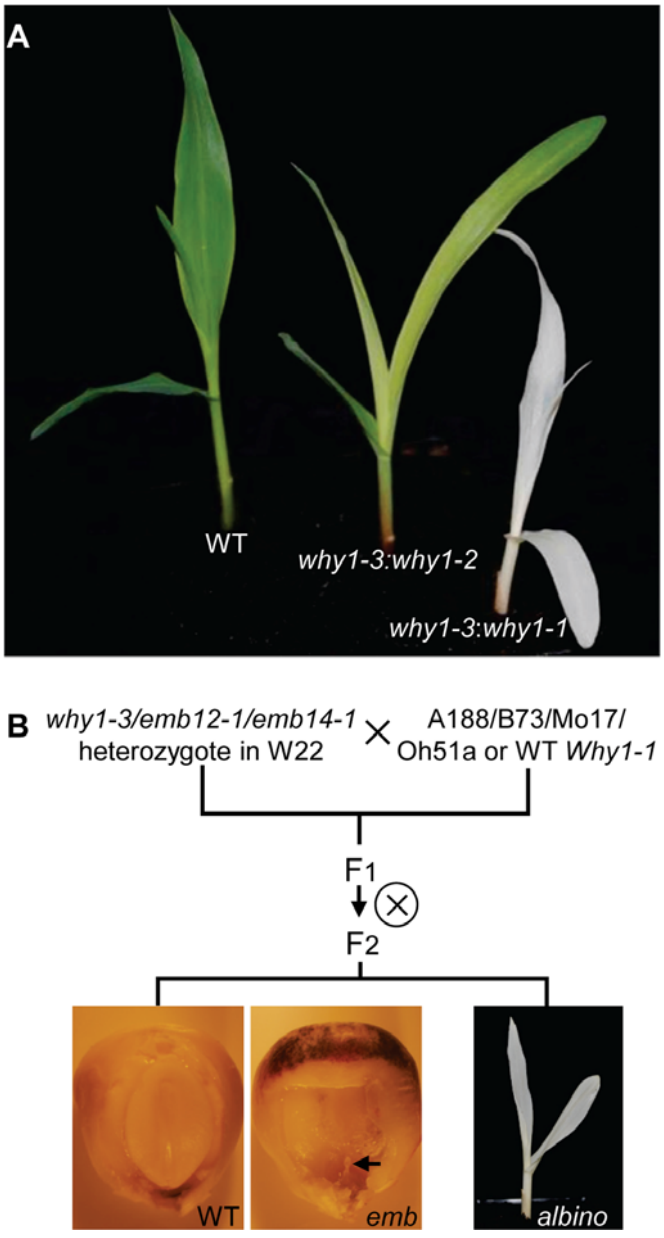


Figure 3.8 Phenotypic expression of *why1-3, emb12-1,* and *emb14-1*. (A) *albino* seedlings from the crosses between heterozygous *why1-3* and *why1-1 or why1-2*. (B) The *emb* kernel and *albino* seedling from the F2 progenies of the crosses between *why1-3*, *emb12-1*, or *emb14-1* heterozygotes in W22 background and A188, B73, Mo17, Oh51a, or WT plant from the *why1-1* segregating line. The arrow points to the defective embryo in *emb* kernel.

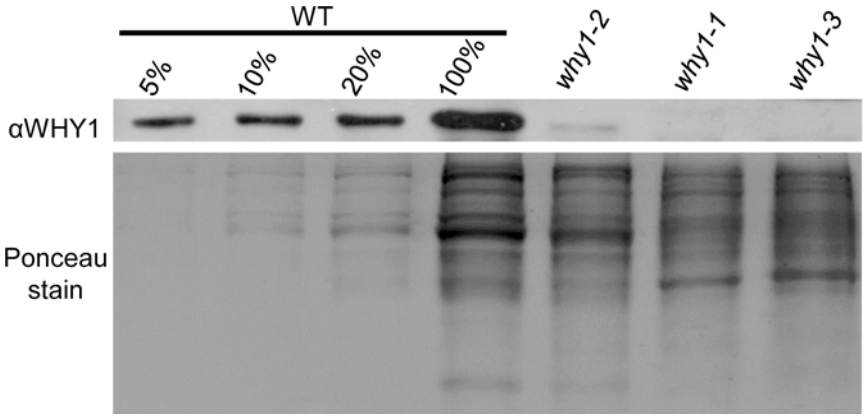


Figure 3.9 Immunoblot analyses on WHY1 protein in the mutant leaf tissue. Total leaf extract of 10 μg protein, or dilutions as indicated were analyzed. The same blot was stained with Ponceau S.

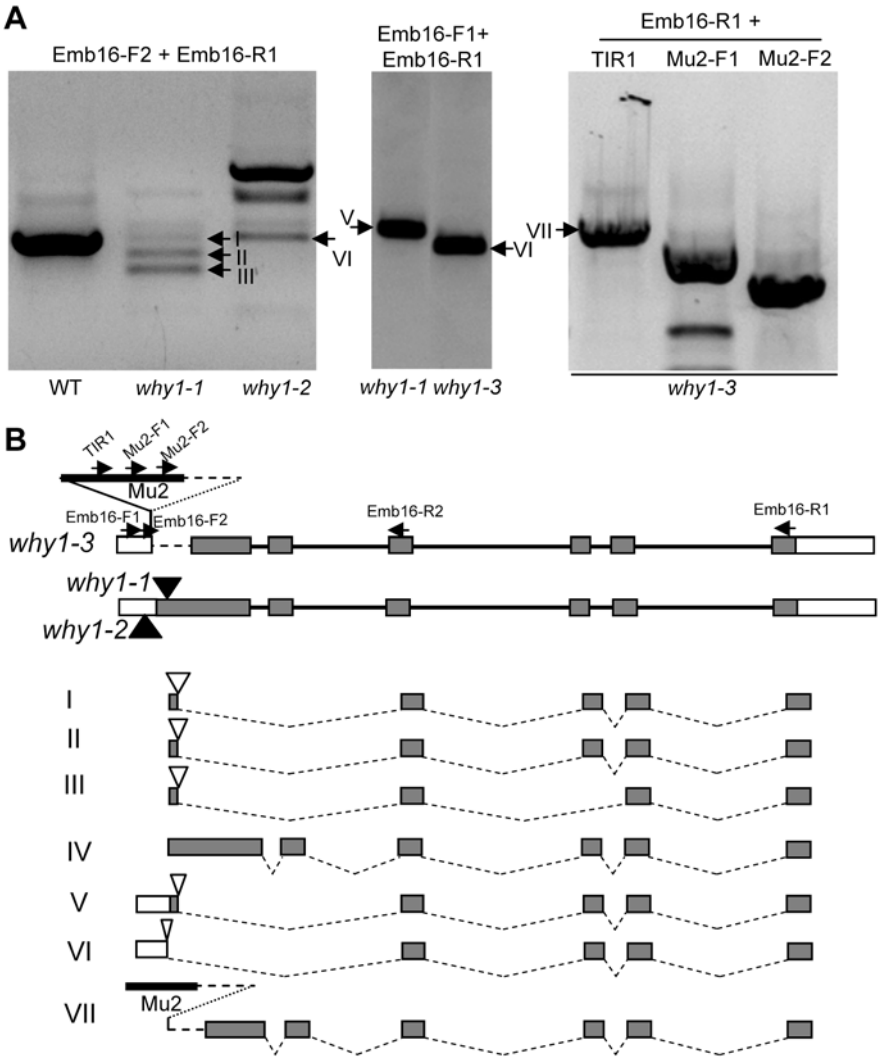


Figure 3.10 Transcriptional studies of *Why1* gene in seedlings homozygous for *whyl-1, why1-2,* or *why1-3*. (A) RT-PCR analysis on WT seedling, and the *albino* seedlings of *why1-1*, *why1-2* and *why1-3* using primers as indicated. TIR1, Mu2-F1, and Mu2-F2 primers are nested primers in *Mu2*. Arrows point to fragments recovered and sequenced. (C) The alternative spliced forms of the *why1* gene in the *albino* seedlings homozygous for *why1-1*, *why1-2*, or *why1-3*. The IV fragment from *why1-2* allele was sequenced to be the WT Why1 gene. The primer sites are indicated by arrows. Empty triangles are the spliced *Mu* element.

(Figure 3.10A). The sequencing result showed it was predicted to be the WT WHY1 protein. In *why1-1* ivory leaves, four major fragments were detected and sequenced (Figure 3.10). The results indicate that these transcripts are all incorrectly spliced, removing a major part of the *MuDR* element and most of the first exon and the entire second exon of *Why1* (Figure 3.10B). None of these transcripts could predict a likely functional WHY1 protein. In the *why1-3* allele, two transcripts were detected (Figure 3.10, A and B). One could not predict a functional protein. The other contained the *Mu2* element which could predict a fusion protein with the N-terminus encoded by the *Mu2* element. However, due to the deletion in the first exon of *Why1* that removed the transit peptide, this fusion protein is unlikely to target itself to the chloroplast. Supporting this conclusion, neither the ChloroP (Emanuelsson *et al*., 1999) nor Predotar (Small *et al*., 2004) algorithms predict a transit peptide in this protein. This analysis suggests that *why1-2* is a weak allele, and *why1-1* and *why1-3* are likely null alleles, which is consistent with the Western blot result. The null nature of *why1-3* mutation is consistent with the deletion in the first exon that removes the transit peptide. However, the *why1-1* allele was reported previously to have a low level expression (Prikryl *et al*., 2008). There were multiple fragments in the RT-PCR analysis of *why1-1* ivory leaves (Figure 3.10A). For that reason, we cannot completely rule out the possibility that the expression level difference contributed to the phenotypic difference.

We then tested whether genetic backgrounds could explain the different phenotypic expression of these alleles. The *why1-3* heterozygotes in the W22 background were crossed with inbreds A188, B73, Mo17, Oh51a and the WT *Why1-1* (Figure 3.8B). The F2 progenies of these crosses segregated *emb* kernels and *albino* seedlings. And reciprocal crosses produced the same result. The ratio of the *emb* and *albino* mutants together is ~25% of all seeds, consistent with one recessive mutation (Table 3.1). The *albino* seedlings were confirmed to be homozygous *why1-3* by PCR genotyping. This result indicates that the *why1-3* mutation could condition *albino*

Table 3.1 Ratio of *emb* kernels plus *albino* seedlings from the F2 progenies of the crosses between *emb* heterozygotes (*why1-3*/+, *emb12-1*/+, and *emb14-1*/+) in W22 background and maize inbred lines (A188, B73 or Mo17), and goodness-of-fit test for a monogenic inheritance.

Crosses in F1 (♀ × ♂)	Total ratio of *emb* and *albino*	Total no. of *emb* and *albino*	Total no. of WT	Expected ratio	x2	P-value
why1-3/+ × A188	26.3%	97	272	1:3	0.26	0.50-0.75
A188 × *why1-3*/+	25.0%	37	111	1:3	0	1
why1-3/+ × B73	29.6%	165	393	1:3	5.97	0.01-0.05
why1-3/+ × Mo17	24.7%	390	1190	1:3	0.07	0.75-0.90
Mo17 × *why1-3*/+	26.4%	288	803	1:3	1.06	0.25-0.50
emb12-1/+ × B73	26.6%	143	395	1:3	0.63	0.25-0.50
emb12-1/+ × Mo17	23.9%	67	213	1:3	0.12	0.50-0.75
emb14-1/+ × B73	23.7%	89	286	1:3	0.26	0.50-0.75
B73 × *emb14-1*/+	27.2%	205	550	1:3	1.75	0.10-0.25
emb14-1/+ × Mo17	25.2%	148	440	1:3	0	>0.95
Mo17 × *emb14-1*/+	23.8%	31	99	1:3	0.04	0.75-0.90

seedling (normal embryogenesis) or embryo lethal phenotype, which is dependent on the genetic backgrounds.

We also crossed *why1-1* allele from its original background to W22 background by continued backcrossing. No *emb* kernels were obtained in the selfed progenies of the first, second and third backcross generations. One possible explanation is that *why1-1* is leaky as indicated previously (Prikryl *et al*., 2008), such that a small amount of WHY1 protein is sufficient for embryogenesis regardless of the genetic background. For that reason, the genetic background dependence of embryogenesis may be masked in the *why1-1* allele. Another possible explanation may rely on the complexity of the genetic background. In the *why1-1* original background, there were more than one loci regulating the phenotypic expression of *why1* mutant, and they are dominant to the ones in W22 inbred. Three times backcross didn't eliminate such dominant loci completely, which is sufficient for embryogenesis in absence of WHY1 protein. The *emb* kernel may be obtained in the F2s of further backcross generations, in which the dominant genetic loci were completely eliminated.

3.7 Plastid translation mutants show genetic background dependence for either *emb* kernel or *albino* seedling phenotypes

One molecular consequence of the *why1-1* mutation is deficiency in plastid ribosome formation, thus the mutant abolishes protein translation of the plastid encoded genes (Prikryl *et al*., 2008). We speculate that the genetic background dependence for embryo lethality may not be unique to *why1*, but a shared feature for other plastid translation mutants as well. To test this notion, we crossed two embryo defective mutations, the *emb12-1* and *emb14-1* heterozygotes with B73 and Mo17 inbreds (Figure 3.8B). The *emb12-1* and *emb14-1* alleles were isolated from the UniformMu population, hence in a nearly isogenic W22 genetic background. *Emb12* encodes the plastid translation initiation factor 3 (Shen *et al*., 2013). *Emb14* encodes an YqeH homolog that shows significant similarity to nitric oxide associated 1 in rice and *Arabidopsis* (Li C. and Tan, B.C., unpublished data). It was believed to function in the ribosome assembly in plastids (Liu *et al*., 2010). Loss of function mutants in

Emb12 or *Emb14* showed a similar embryo arrest as the *why1-3*. Also similar with *why1-3*, both *emb* kernels and *albino* seedlings were obtained in the F2 progenies of all the crosses (Figure 3.8B), and the ratio of *emb* plus *albino* mutants was ~25% (Table 3.1). Reciprocal crosses showed the same result. PCR genotyping confirmed the *albino* seedlings were homozygous for *emb12-1* or *emb14-1* allele. These results indicate that the *emb12* and *emb14* mutations also condition *albino* phenotype in B73 and Mo17 backgrounds, thus confirms that the requirement of plastid translation for embryogenesis in maize is determined by the genetic background.

3.8 More than one genetic loci regulate embryogenesis in plastid translation mutants

We also characterize the genetic factors regulating embryogenesis in plastid translation mutants. In the F2 generations of the crosses between *why1-3* heterozygotes in W22 background and A188, B73, Mo17, or Oh51a inbreds, *emb* embryos from the segregating ears were arrested at or beyond the transition stage (Figure 3.11A). The germinating kernels from a same ear gave rise to WT green seedling, and *albino* seedlings with either normal or abnormal leaves (Figure 3.11B). And there is a gradient in the abnormality regarding the formation of coleoptile and leaves. The PCR genotyping confirms the normal and abnormal *albino* seedlings are homozygous for *why1-3* allele. Similar results were observed with *emb12-1* and *emb14-1* mutants. Given coleoptile and the first 4 to 6 primary leaves have been formed during embryogenesis in normal maize seeds (Figure 3.2G), these results indicate embryogenesis in absence of plastid translation is likely arrested consecutively from the transition stage to the formation of leaf primordial. If there is one genetic locus regulating embryogenesis in the plastid translation mutants, there will be only two types of phenotypes with the lethal embryos arrested at the transition stage and the *albino* seedlings with normal morphology in F2 progenis of these crosses. Thus, these results indicate there should be more than one genetic loci, which interacting with the genes essential for the biogenesis of plastid translation machinery to regulate embryogenesis in maize.

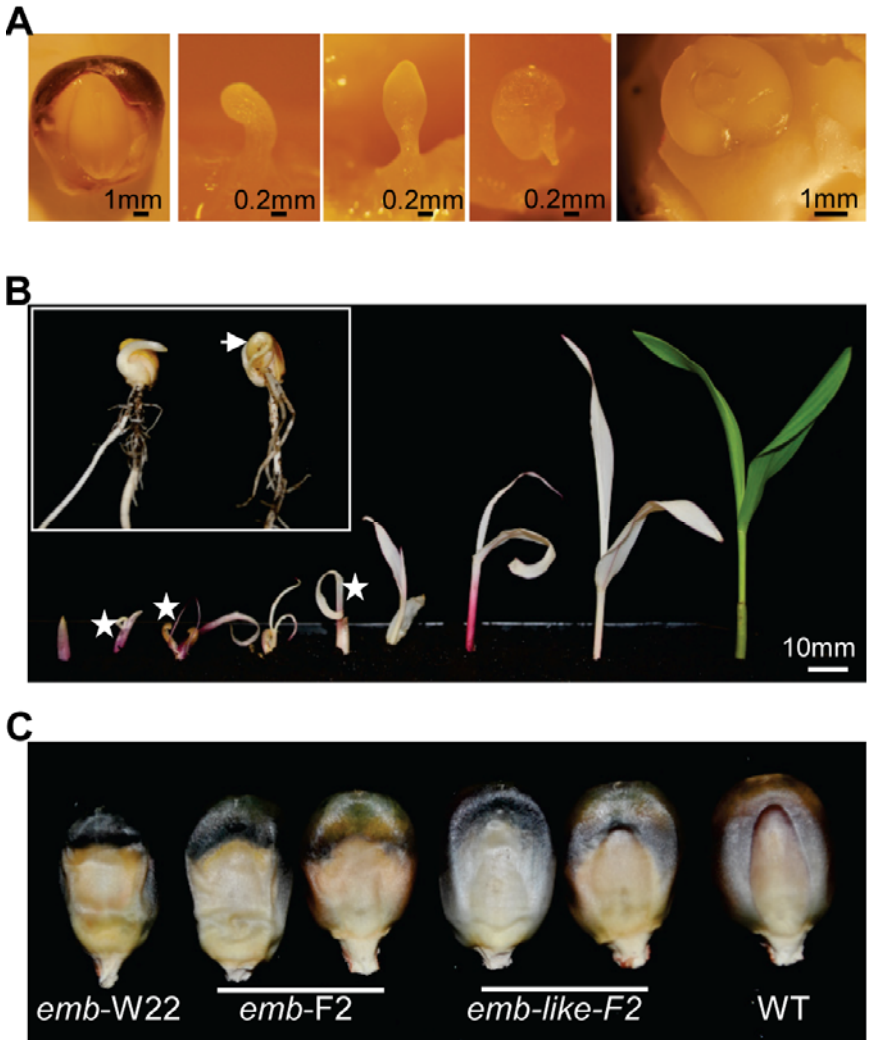


Figure 3.11 Different types of *emb* kernels and *albino* seedlings from the F2 of the crosses between *why1-3* in W22 background and Mo17 inbred line.

(A) 20DAP *emb* kernels arrested at different developmental stages of embryogenesis. (B) *albino* seedlings with normal and abnormal formation of leaves. All of these *albino* seedlings have been genotyped to be *emb16* mutant. White arrow points to the abnormal albino seedling in the enlarged figure. (C) Mature *emb* kernels from F2 of the cross with Oh51A inbred (Oh51A) compared with typical *emb16* kernel in W22 background (W22). Scale bars as indicated.

Corresponding to the consecutively abortion in embryo development, both typical *emb* kernels and kernels similar to *emb* kernels (*emb-like*) were observed (Figure 3.11C). To determine the ratio of *albino* seedlings and *emb* kernels with embryo lethality, we sowed all *emb* and *emb-like* kernels. Some of the *emb-like* kernels germinated and gave rise to *albino* seedlings with abnormal leaves, and the others didn't germinate. Those ungerminated *emb-like* kernels were considered to be *emb* kernels which contain lethal embryos. By this way, the number of *emb* and *albino* phenotypes was calculated (Table 3.1). When crossed with Mo17, the ratio of *emb* and *albino* in *why1-3* is about 13.8% and 11.3%, respectively; in *emb12-1* and *emb14-1*, most of the mutants condition *emb* phenotype (23.4% and 22.9%, respectively), and only a small part conditions *albino* phenotype (0.4% and 2.1%, respectively). When crossed with B73, the *emb* ratio of *why1-3*, *emb12-1*, and *emb14-1* mutants are 16.5%, 14.4%, and 12.5%, respectively, and there is a high variation for *why1-3* and *emb12-1* mutants, suggesting an environmental effect. The continued backcross of *why1-3* and *emb12-1* from W22 to B73 background reduces the ratio of *emb* kernels. In the selfed progenies of the third backcross generation, the ratio of *emb* kernels for *why1-3* and *emb12-1* is reduced to 4% and 3%, respectively. These results indicate the *emb* phenotype conditioned by the plastid translation mutations is being suppressed by increasing the dosage of B73 genome. By contrast, the *albino* phenotype is suppressed in the W22 background. In another word, the genetic loci regulating embryogenesis are present at two extremes between W22 and B73 inbred lines. In absence of plastid translation, they arrest embryogenesis at the transition stage in W22 background, but allow normal embryogenesis in B73 background. Due to the limited population size, we cannot point out the number of the genetic loci.

3.9 Plastid development is impaired in *why1* mutant

The function of WHY1 in maize has not been well understood although several studies have been reported (Cappadocia *et al*., 2010; Prikryl *et al*., 2008). The

chloroplast localization of WHY1 promoted us to examine the impact on plastid biogenesis in the absence of WHY1 protein.

By transmission electron microscopy (TEM), the ultrastructure of proplastids in the embryos from *why1-3* allele in W22 background was analyzed. At 6 DAP, proplastids in the WT embryo contain almost no inner membrane structure, and this feature is quite common to proplastids in the embryo proper cells (Figure 3.12A; Appendix 1.1A). By contrast, the inner structure of proplastids in the *why1-3* mutant is varied, some with thylakoids and/or vesicle-like structures, others with starch granules (Figure 3.12E; Appendix 1,1D). From 7 to 14 DAP, the inner structure in proplastids developed with increased membrane system as the WT embryo differentiated (Figure 3.12, B-D; Appendix 1.1, B and C). By 14 DAP, the linear thylakoid has been formed (Figure 3.12D). During the same developmental stages, no significant change in the structure of proplastid was observed in mutant embryos (Figure 3.12, F-H; Appendix 1.1, E and F). In addition to the abnormal development of proplastids, there is also a defect in the development of mitochondria was. Although no striking differences in the structure of mitochondria and mitochondrial size were observed, the number of mitochondria per cell was much higher in the mutant embryos from 6 to 8 DAP (Figure 3.12, E-G; Appendix 1.1 D-F). At 14 DAP, the high number of mitochondria disappeared, and the mutant embryo cells become vacuolated (Figure 3.12H), a sign of cell death (Dominguez *et al*., 2001; Magnard *et al*., 2004). These observations suggest that the *why1* mutation in W22 background causes abnormal formation of the inner structure of proplastids, and intriguingly an increased number of mitochondria. The mutant embryo cells undergo cell death at 14 DAP, which is the reason of the failure in the embryo rescue experiment on *why1-3* embryos.

By changing the genetic background, the *why1-3* mutant was rescued and underwent the embryogenesis process normally. So, we studied the biogenesis of chloroplast and mitochondrion in the *albino* leaves of *why1-3* mutant, as well as *why1-1* and *why1-2* alleles (Figure 3.13). When compared with the WT, the thylakoid

biogenesis was found impaired in chloroplasts of all three alleles, and the severity of the impact was consistent with severity of mutation, *i.e.* more severely arrested in *why1-1* and *why1-3* alleles than in the *why1-2* leaky allele (Figure 3.13, A-H). In *why1-2*, most of the thylakoids were stacked to form grana in mesophyll cells, but no grana formation was observed in *why1-1* and *why1-3*, which was due to the low amount of thylakoid. In contrast to the increased number of mitochondria in the mutant embryo cells of *why1-3*, the number of mitochondria in the leaf cells was comparable between the mutants and the WT (data not shown). Moreover, no difference in the structure and size of mitochondria was observed. These results suggest (1) WHY1 is required for the biogenesis of the inner membrane system in plastids, but (2) may negatively regulate the stacking of thylakoids to form granas; (3) the difference in the number of mitochondria between *why1-3 emb* embryos (W22 background) and *albino* leaves (50% W22 and 50% B73 background) may be due to the different status of the genetic loci regulating phenotypic expression of *why1* mutants in the background.

Similar to *why1-1* and *why1-3*, the biogenesis of thylakoid was also severely reduced in the *albino* seedlings of *emb14-1* and *ij* (Figure 3.13, I-L; Coe *et al*., 1988). Since IJ protein is also required for plastid translation (Prikryl *et al*., 2008), these results further suggest that plastid translation is required for biogenesis of thylakoid membranes.

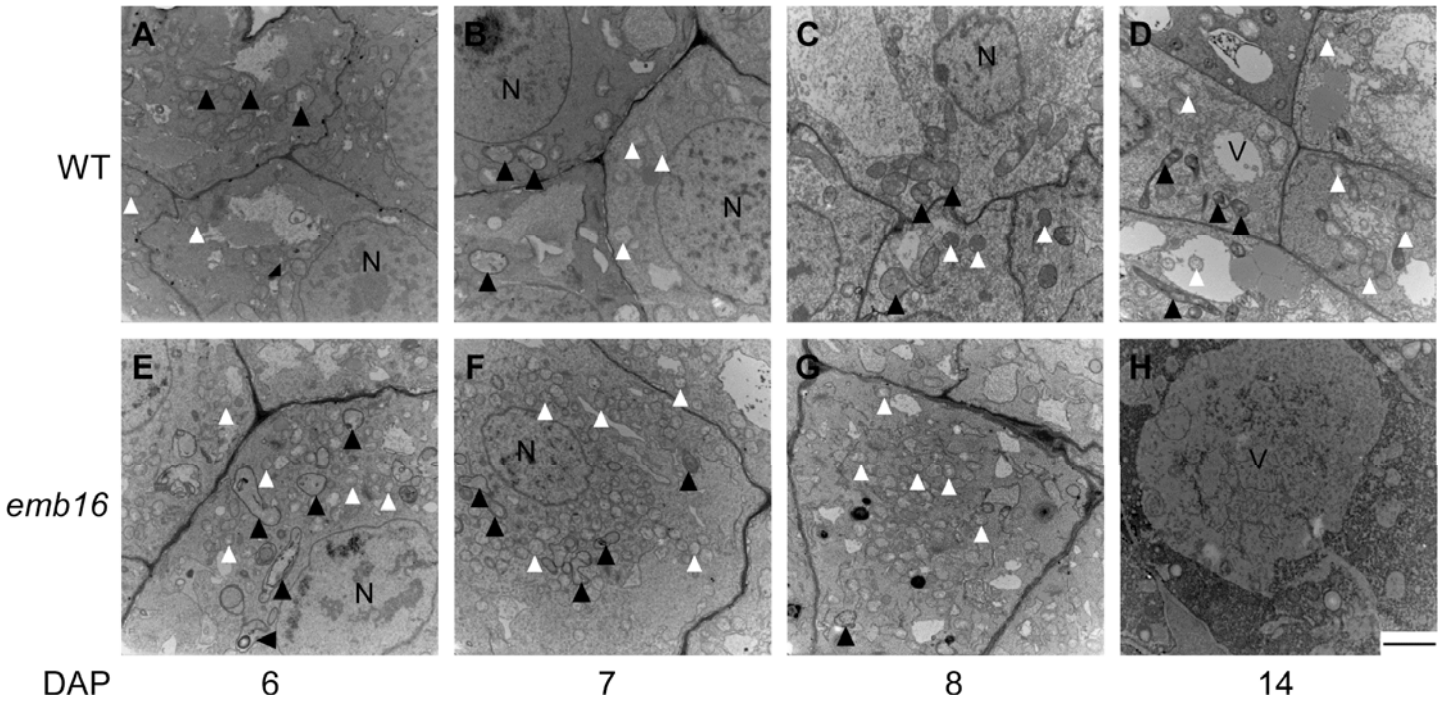


Figure 3.12 Embryo cell development in *emb16* mutant. The WT and *emb16* mutant kernels from segregating ears were sectioned from 6 to 14 DAP. At 6 DAP, the *emb16* embryo was distinguished from the WT by the size and structure of embryo proper using stereomicroscopy and confirmed by the endosperm genotyping. The ultrastructural observation of embryo cells in the *emb16* mutant is from the embryo proper cells and in the WT is from the embryo proper cells (6 DAP) or shoot meristem cells (7-14 DAP). The content of the embryo proper cells is different from that of suspensor cells, which contain more starch granules and vacuoles. Similar cell contents were observed in cells of WT shoot meristem, leaf primordia, and coleoptile. Empty arrow heads point to mitochondria, and filled arrow heads point to proplastids. N: nucleus, V: vacuole. Scale bars =2µm.

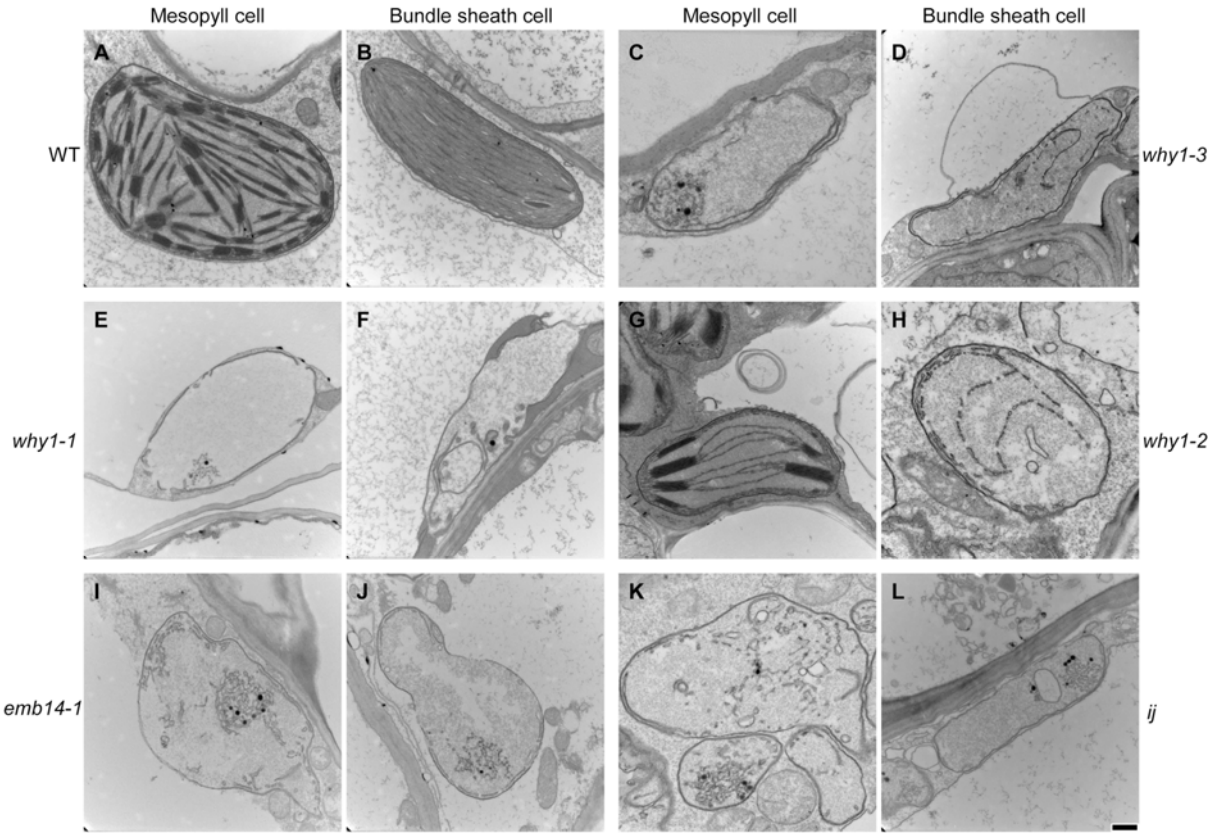


Figure 3.13 Chloroplast in seedlings homozygous for *why1-1*, *why1-2*, *why1-3*, *emb14-1*, or *ij*. Leaf sector 1 cm below the tip of the second leaf from two-leaf stage seedlings were fixed and sectioned for TEM. Scale bars=0.5 μm.

Chapter 4 Discussion

Through the molecular characterization, we revealed that the arrest of embryogenesis in the maize *emb16* mutant is caused by a mutation in *Why1* gene, thus demonstrating that embryogenesis requires the function of *Why1*. WHY1 has been implicated in modulating the homeostasis of telomere length and activating or repressing transcription in the nucleus (Desveaux *et al*., 2000, 2004; Xiong *et al*., 2009; Yoo *et al*., 2007), and genome stability and ribosome formation in the plastid (Cappadocia *et al*., 2010; Prikryl *et al*., 2008). However, its molecular function is still unclear. Genetic analyses revealed that the requirement of WHY1 function for embryogenesis can be suppressed in maize A188, B73, Mo17 and Oh51a inbred, giving rise to an *albino* seedling phenotype. And similar suppression was found in *emb12* and *emb14* mutants which were impaired in plastid translation process. Given that *why1* mutants are deficient in plastid ribosomes (Prikryl *et al*., 2008), these results indicate that the requirement of plastid translation for embryogenesis can be suppressed by a likely common mechanism.

4.1 Embryogenesis in maize is regulated by a new pathway related with plastid translation but independent of the plastid-encoded genes

The genetic suppression of embryo lethality by genetic background offers an explanation to the relationship between plastid translation and embryogenesis. In *Arabidopsis*, mutations impairing plastid translation process cause embryo lethality (Bryant *et al*., 2011; Romani *et al*., 2012; Cushing *et al*., 2005). However, in maize, mutations abolishing plastid ribosome assembly and translation gave rise to three phenotypes, *i.e.* lethal embryo, *albino* seedling and stripped leaves (Ma and Dooner 2004; Magnard *et al*., 2004; Shen *et al*., 2013; Williams and Barkan 2003; Schmitz-Linneweber *et al*., 2006; Beick *et al*., 2008; Khrouchtchova *et al*., 2012; Prikryl *et al*., 2008; Coe *et al*., 1998). The first class of mutants demonstrates an essential function of plastid translation to embryogenesis. Whereas the last two classes

of mutants indicate that embryogenesis does not require the expression of the entire plastid genome because all these mutants have normal embryogenesis and produce viable seeds. Given our results on *why1*, *emb12* and *emb14*, it is likely that the *albino* seedling and striping leaf phenotypes are conditioned in specific genetic backgrounds where embryo lethality is suppressed. Previous studies of the plastid ribosome deficient leaf striping mutant *ij* have revealed evidence of genetic suppressors (Prikryl *et al*., 2008; Coe *et al*., 1998). In K55 and Ky21 genetic backgrounds, *ij* conditions embryo lethality, but seedlings with stripe leaves in Mo17 and Oh51a backgrounds. Similar suppression was observed in *emb8522*, which conditions embryo lethality in the original genetic background but in A188 and B73 backgrounds conditions *albino* seedlings (Sosso *et al*., 2012). *Emb8522* encodes a plastid PPR protein with possible functions in plastid gene expression. Together, these results suggest that the genetic background difference in maize is a key factor that contributes to the unequal requirement of plastid translation for embryogenesis in maize and *Arabidopsis*.

This genetic background determination of the requirement of plastid translation for embryogenesis implies a genetic mechanism mediating this process. One puzzle in embryogenesis in flowering plants is to understand what factors constitute the requirement of plastid translation for embryogenesis. One hypothesis assumes that specific products encoded by the plastid genome are required for embryogenesis (Bryant *et al*., 2011; Sosso *et al*., 2012), and another hypothesis assumes that a defect in plastid protein translation triggers the release of a retrograde signal to shut down the embryogenesis process (Shen *et al*., 2013). These two hypotheses are not mutually exclusive. In *Arabidopsis*, the *accD*, *clpP1*, *ycf1* and *ycf2* genes in the plastid genome have been considered (Bryant *et al*., 2011; Kuroda and Maliga 2003; Drescher *et al*., 2000). The *accD* gene encodes the β-CT subunit of the plastid He-ACCase, which is required for the biosynthesis of fatty acid; *clpP1* encodes a proteolytic subunit of the *clp* protease, which regulates protein degradation in plastids. In Arabidopsis, the fatty acid synthesis pathway and protein degradation via Clp protease system are demonstrated to be essential for embryogenesis (Kim *et al*., 2009, 2013; Li *et al*.,

2011). In maize, the *accD*, *ycf1* and *ycf2* genes no longer exist in the plastid genome, and yet defective plastid translation mutations still cause embryo lethality (Ma and Dooner 2004; Magnard et al., 2004; Shen *et al*., 2013; Maier *et al*., 1995). This evidence argues that these genes and the related cellular processes are not determinant factors for embryogenesis, at least in maize. Although *clpP1* gene is present in the maize plastid genome, the requirement of *clpP1* for embryogenesis was also challenged. This is because (1) unedited *clpP1* did not cause embryo lethality (Chateigner-Boutin *et al*., 2008), (2) the elimination of *clpP1* from maize plastid genome didn't affect cell viability (Cahoon *et al*., 2003), and (3) the embryogenesis in mazie *albino* mutant defective in plastid translation proceeds normally (Williams and Barkan, 2003; Prikryl *et al*., 2008). Sosso *et al*. (2012) proposed that the plastid *trnE* gene encoding tRNA-Glu may be the plastid factor. Besides protein translation, tRNA-Glu is the substrate for haem synthesis and haem is an essential prosthetic group of many important proteins in plastids and mitochondria. However, haem biosynthetic mutants did not condition embryo lethality in maize and *Arabidopsis* (Woodson and Chory, 2011; Ishikawa *et al*., 2001*)*.

Our results favor the retrograde signaling hypothesis. The suppression of embryo lethality in *why1*, *emb12* and *emb14* in certain genetic backgrounds suggests the presence of suppressor(s) that can suppress the requirement of plastid translation for embryogenesis, and the suppressor(s) are encoded by more than one genetic loci. Maize is known for its wide diversity (Springer *et al*., 2009; Stupar and Springer 2006; Schnable *et al*., 2009). Inbred lines B73 and Mo17 are different in copy numbers in several hundred sequences and presence/absence variations in several thousand sequences (Springer *et al*., 2009), and 4-18% genes with differential expression patterns (Stupar and Springer 2006). This diversity renders the possibility that a functional homolog with overlapping expression of *Why1* in certain inbreds that confers the suppressor function. We reason this possibility is unlikely because: 1) we did not find another copy of *Why1* in the sequenced B73 genome; 2) *Why1* (chr6), *Emb12* (chr5) and *Emb14* (chr4) locate on different chromosomes. Thus, all three

genes should have at least one paralog or one homolog in the B73 genome, for which we did not find in the sequenced genome. Overwhelming evidence supports the existence of a retrograde signaling pathway coordinating the plastid and the nuclear gene expression (Woodson and Chory 2008). This signal may be associated with the plastid translation machinery to monitor its integrity. A defect in plastid translation machinery triggers its release and a shut-down of the cell activity, thus causing embryo lethality. Along with this reasoning, this pathway may be fully functional in the W22 genetic background but dysfunctional in A188, B73, Mo17 and Oh51a genetic backgrounds as a result of natural mutations. This would provide a plausible explanation for the suppression of embryo lethality in some maize genetic backgrounds, but not in others.

4.2 The unequal requirement of plastid translation for embryo and endosperm development in maize

The *why1-3* allele in the W22 genetic background conditions specific arrest in embryogenesis without major impact on endosperm development. This phenotype is found in *lem1*, *emb8516* and *emb12* mutant (Ma and Dooner 2004; Magnard *et al*., 2004; Shen *et al*., 2013), all of which are implicated in plastid translation machinery. Comparing with the arrested embryogenesis, the endosperm development appears to be less dependent on the plastid gene expression in these mutants regardless of the genetic backgrounds. This difference may be related to the different fates of proplastids in the endosperm and the embryo (Shen *et al*., 2013). Considering the results of this study, it is also possible that the retrograde signaling pathway in the embryo is not present in the endosperm. As such, the endosperm does not produce the signal even though the plastid translation is defective.

4.3 *emb16* mutant embryos cannot be rescued

The *emb16* embryos from 7 to 20 DAP cannot be rescued, which may be due to the failure in the formation of shoot meristem. This result is inconsistent with the embryo rescue results from another three *emb* mutants, i.e. *emb*-7191*, *emb*-7182*,

emb-7192*. In their original background, *emb*-7191*, *emb*-7182*, *emb*-7192* were arrested at the late proembryo or the transition stage (Consonni *et al*., 2003). By culturing of the mutant embryos from F2s of the crosses with W64A inbred, a viariable ratio of mutant embryos were rescued, giving rise to the *albino* seedling. The anthors explained the success in embryo rescue experiments is due to the induction of shoots in the mutant embryos by MS medium (Consonni *et al*., 2003). In this study, we know that *emb* mutants defective in plastid translation can go beyond the transition stage to form shoot meristem by changing their genetic backgrounds in to A188, B73, Mo17, or Oh51a inbred lines. So, the reason that *emb*-7191*, *emb*-7182*, *emb*-7192* rescued may lie in the same mechanism. In the F2s of the crosses with W64A inbred, the *emb* embryos can go beyond the transition stage to form shoot and root meristem, which makes the embryo rescue on MS medium much easier. So, the success of the embryo rescue experiments on F2s of the three *emb* mutants lie in the formation of shoot meristem during embryogenesis, not the induction of SAM by the MS medium. Similar with A188, B73, Mo17, and Oh51A, the W64A inbred may not contain the genetic suppressors. By contrast, the original background of *emb*-7191*, *emb*-7182*, *emb*-7192* may be similar with W22 inbred, containing the embryogenesis surpressors.

4.4 The role of maize ACR1 in plant development

Due to the lack of *acr* mutants, the role of ACR proteins in plant development is still not clear. The *acr1* mutation derived from *emb16* mutant may be a weak allele, with a *Mu8* insertion 69 bps upstream of the translation start codon. Since the expression of this mutant allele was not checked, we are not sure the normal growth of *acr1* mutant is due to the normal expression of *ACR1* gene, or the non-essential role of ACR1 protein in plant development. It is necessary to study the acr mutant for of role of ACR proteins in plant development.

Chapter 5 Conclusion and perspective

The *embryo defective16* (*emb16*) mutant is a typical *emb* mutant in maize, which was isolated from the UniformMu population in near isogenic W22 genetic background (McCarty *et al*., 2005). The mutation causes embryo development arrest at transition stage, but allows endosperm development almost normal. Molecular cloning indicates that the *emb* phenotype is caused by a null mutation of the *Why1* gene. Further genetic analyses demonstrate that the requirement of WHY1 function for embryogenesis is dependent on the genetic background. And this dependence exists in two other embryo defective genes (*emb12* and *emb14*) that affect plastid translation. These results indicate that the requirement of plastid translation for embryogenesis may not be related with the expression of maize plastid genome, and reveals the presence of a pathway regulating embryogenesis in maize, which is independent of the *accD* pathway. This new pathway is regulated by some unknown genetic factors that are encoded by more than one genetic loci, and these genetic loci are present differently between W22 and A188, B73, Mo17, and Oh51a inbreds. Identifying these genetic loci is the key to understanding the plastid function in embryogenesis and plant development.

References

Abbe, E. C. and Stein, O. L. 1954. The growth of the shoot apex in maize: Embryogeny. *American Journal of Botany* **41**: 285-293.

Aravind, L. and Koonin, E. V. 1999. Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches. *Journal of Molecular Biology* **287**: 1023–1040.

Arcondéguy, T., Jack, R. and Merrick, M. 2001. PII signal transduction proteins, pivotal players in microbial nitrogen control. *Microbiology* and Molecular *Biology* Reviews **65**: 80–105.

Asakura, Y. and Barkan, A. 2006. *Arabidopsis* orthologs of maize chloroplast splicing factors promote splicing of orthologous and species-specific group II introns. *Plant Physiology* **142**: 1656-1663.

Babiychuk, E., Vandepoele, K., Wissing, J., Garcia-Diaz, M., De Rycke, R., Akbari, H., Joubès, J., Beeckman, T., Jänsch, L., Frentzen, M., Van Montagu, M. C. and Kushnir, S. 2011. Plastid gene expression and plant development require a plastidic protein of the mitochondrial transcription termination factor family. Proceedings of the National Academy of Sciences **108**: 6674-6679.

Beick, S., Schmitz-Linneweber, C., Williams-Carrier, R., Jensen, B. and Barkan, A. 2008. The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. *Molecular and Cellular Biology* **28**: 5337-5347.

Bellaoui, M., Keddie, J. S. and Gruissem, W. 2003. DCL is a plant-specific protein required for plastid ribosomal RNA processing and embryo development. *Plant Molecular Biology* **53**: 531-543.

Bennetzen, J. and Hake, S. C. (Eds.) 2009. *Handbook of Maize: Its Biology*. Springer-Verlag, New York.

Benning, C., and Ohta, H. 2005. Three enzyme systems for galacto-glycerolipid biosynthesis are coordinately regulated in plants.*The Journal of Biological Chemistry* **280**: 2397-2400.

Block, M. A., Dorne, A. J., Joyard, J. and Douce, R. 1983. Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. II. Biochemical characterization. *The Journal of Biological Chemistry* **258**: 13281-13286.

Bloom, M., Evans, E. and Mouritsen, O. G. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Quarterly Reviews of Biophysics* **24**: 293-397.

Bryant, N., Lloyd, J., Sweeney, C., Myouga, F. and Meinke, D. 2011. Identification of nuclear genes encoding chloroplast-localized proteins required for embryo development in *Arabidopsis*. *Plant Physiology* **155**: 1678-1689.

Cahoon, A. B., Cunningham, K. A. and Stern, D. B. 2003. The plastid clpP gene may not be essential for plant cell viability. *Plant Cell Physiology* **44**: 93-95.

Cappadocia, L., Marechal, A., Parent, J. S., Lepage, E., Sygusch, J. and Brisson, N. 2010. Crystal structures of DNA-Whirly complexes and their role in *Arabidopsis* organelle genome repair. *The Plant Cell* **22**: 1849-1867.

Chateigner-Boutin, A. L., Ramos-Vega, M., Guevara-García, A., Andrés, C., de la Luz Gutiérrez-Nava, M., Cantero, A., Delannoy, E., Jiménez, L. F., Lurin, C., Smal,l I. and León, P. 2008. CLB19, a pentatricopeptide repeat protein required for editing of rpoA and clpP chloroplast transcripts. *The Plant Journal* **56**:590-602.

Chen, J. and Dellaporta, S. L. 1994. Urea-based plant DNA miniprep. In M Freeling, and V Walbot (eds). *The Maize Handbook*. New York: Springer-Verlag. pp. 526-527.

Clark, J. K. and Sheridan, W. F. 1991. Isolation and characterization of 51 embryo-specific mutations of maize. *The Plant Cell* **3**: 935-951.

Cline, K. 1986. Import of Proteins into Chloroplasts. *Journal of Biological Chemistry* **261**: 14804-14810.

Coe, E. H., Thompson, D. and Walbot, V. 1988. Phenotypes mediated by the iojap genotype in maize. *American Journal of Botany* **75**: 634-644.

Consonni, G., Aspesi, C., Barbante, A., Dolfini, S., Giuliani, C., Giulini, A., Hansen, S., Brettschneider, R., Pilu, R. and Gavazzi, G. 2003. Analysis of four maize mutants arrested in early embryogenesis reveals an irregular pattern of cell division. *Sexual Plant Reproduction* **15**:281-290

Cushing, D. A., Forsthoefel, N. R., Gestaut, D. R. and Vernon, D. M. 2005. *Arabidopsis* emb175 and other ppr knockout mutants reveal essential roles for pentatricopeptide repeat (PPR) proteins in plant embryogenesis. *Planta* **221**: 424-436.

DellaPenna, D. and Pogson, B. J. 2006. Vitamin synthesis in plants: tocopherols and carotenoids. *Annual Review of Plant Biology* **57**: 711-738.

Desveaux, D., Despres, C., Joyeux, A., Subramaniam, R. and Brisson, N. 2000. PBF-2 is a novel single-stranded DNA binding factor implicated in PR-10a gene activation in potato. *The Plant Cell* **12**: 1477-1489.

Desveaux, D., Subramaniam, R., Despres, C., Mess, J. N., Levesque, C., Fobert, P. R., Dangl, J. L. and Brisson, N. 2004. A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. *Developmental Cell* **6**: 229-240.

Ding, C., Ma, D., Wang, S. and Ding, Y. 2011. Optimization process and method of 2-D electrophoresis for rice proteomics. *Chinese Bulletin of Botany* **46**: 67-73.

Dominguez, F., Moreno, J. and Cejudo, F. J. 2001. The nucellus degenerates by a process of programmed cell death during the early stages of wheat grain development. *Planta* **213**: 352-360.

Drescher, A., Ruf, S., Calsa, T., Carrer, H. and Bock, R. 2000. The two largest chloroplast genome-encoded open reading frames of higher plants are essential genes. *The Plant Journal* **22**: 97-104.

Dyall, S. D., Brown, M. T. and Johnson, P. J. 2004. Ancient invasions: from endosymbionts to organelles. *Science* 304: 253-257.

Emanuelsson, O., Nielsen, H. and von Heijne, G. 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Science* **8**: 978-984.

FAO: www.faostat.fao.org.

Frick, G., Su, Q., Apel, K. and Armstrong, G. A. 2003. An *Arabidopsis porB porC* double mutant lacking light-dependent NADPH:protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested *The Plant Journal* **35**:141-153.

Grabowski, E., Miao, Y., Mulisch, M., Krupinska, K. 2008. Single-stranded DNA-binding protein Whirly1 in barley leaves is located in plastids and the nucleus of the same cell. *Plant Physiology* **147**: 1800-1804.

Hayakawa, T., Kudo, T., Ito, T., Takahashi, N. and Yamaya, T. 2006. ACT domain repeat protein 7, ACR7, interacts with a chaperone HSP18.0-CII in rice nuclei. *Plant Cell Physiology* **47**:891-904.

Hsieh, M. H. and Goodman, H. M. 2002. Molecular characterization of a novel gene family encoding ACT domain repeat proteins in Arabidopsis. *Plant Physiology* **130**:1797-1806.

Hsu, S. H., Belmonte, M. F., Harada, J. J. and Inoue, K. 2010. Indispensable roles of plastids in *Arabidopsis thaliana* embryogenesis. *Current Genomics* **11**: 338-49.

Ishikawa, A., Okamoto, H., Iwasaki, Y. and Asahi, T. 2001. A deficiency of coproporphyrinogen III oxidase causes lesion formation in *Arabidopsis*. *The Plant Journal* **27**: 89-99.

Johansen, D. A. 1940. *Plant Microtechnique*. New York and London: McGraw-Hill Book Company, Inc. pp. 80-82.

Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J., and Rolland, N. 2010. Chloroplast proteomics highlights the subcellular compartmentation of lipid metabolism. *Progress in Lipid Research* **49**: 128-158.

Kesari, R., Lasky, J. R., Villamor, J. G., Des Marais, D. L., Chen, Y. J., Liu, T. W., Lin, W., Juenger, T. E. and Verslues, P. E. 2012. Intron-mediated alternative splicing of *Arabidopsis* P5CS1 and its association with natural variation in proline and climate adaptation. *Proceedings of the National Academy of Sciences* **109**: 9197-9202.

Kessler, F. and Schnell, D. 2009. Chloroplast biogenesis: Diversity and regulation of the protein import apparatus. *Current Opinion in Cell Biology* **21**: 494-500.

Khrouchtchova, A., Monde, R. A. and Barkan, A. 2012. A short PPR protein required for the splicing of specific group II introns in angiosperm chloroplasts. *RNA* **18**: 1197-1209.

Kim, J., Olinares, P. D., Oh, S., Ghisaura, S., Poliakov, A., Ponnala, L. and van Wijk, K. L. 2013. Modified Clp protease complex in the clpP3 null mutant and consequences for chloroplast development and function in *Arabidopsis*. *The Plant Physiology* **162**: 157-179.

Kim, J., Rudella, A., Ramirez, R. V., Zybailov, B., Olinares, P. D. and van Wijk, K. J. 2009. Subunits of the plastid ClpPR protease complex have differential contributions to embryogenesis, plastid biogenesis, and plant development in *Arabidopsis*. *The Plant Cell* **21**: 1669-1692.

Kobayashi, K., Kondo, M., Fukuda, H., Nishimura, M. and Ohta, H. 2007. Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis. *Proceedings of the National Academy of Sciences* **104**: 17216–17221.

Kode, V., Mudd, E. A., Iamtham, S. and Day, A. 2005. The tobacco plastid accD gene is essential and is required for leaf development. *The Plant Journal* **44**:237-244.

Konishi, T. and Sasaki, Y. 1994. Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance toward herbicides. *Proceedings of the National Academy of Sciences* **91**: 3598-3601.

Koussevitzky, S., Stanne, T. M., Peto, C. A., Giap, T., Sjögren, L. L., Zhao, Y., Clarke, A. K. and Chory, J. 2007. An *Arabidopsis thaliana* virescent mutant reveals a role for ClpR1 in plastid development. *Plant Molecular Biology* **63**: 85-96.

Krause, K., Kilbienski, I., Mulisch, M., Rodiger, A., Schafer, A. and Krupinska, K. 2005. DNA-binding proteins of the Whirly family in *Arabidopsis* thaliana are targeted to the organelles. *FEBS Letters* **579**: 3707-3712.

Kudo, T., Kawai, A., Yamaya, T. and Hayakawa, T. 2008. Cellular distribution of ACT domain repeat protein 9, a nuclear localizing protein, in rice (Oryza sativa). *Physiologia Plantarum* **133**: 167–179.

Kuroda, H. and Maliga, P. 2003. The plastid clpP1 protease gene is essential for plant development. *Nature* **425**:86-89.

Li, X., Ilarslan, H., Brachova, L., Qian, H., Li, L., Che, P., Wurtele, E. S. and Nikolau, B. J. 2011. Reverse-genetic analysis of the two biotin-containing subunit genes of the heteromeric aetyl-coenzyme A carboxylase in *Arabidopsis* indicates a unidirectional functional redundancy. *Plant Physiology* **155**: 293–314.

Liu, H., Lau, E., Lam, MP., Chu, H., Li, S., Huang, G., Guo, P., Wang, J., Jiang, L., Chu, I. K., Lo, C. and Tao, Y. 2010. OsNOA1/RIF1 is a functional homolog of AtNOA1/RIF1: implication for a highly conserved plant cGTPase essential for chloroplast function. *New Phytologist* **187**: 83-105.

Liu, Q. P. 2006. Computational identification and systematic analysis of the *ACR* gene family in Oryza sativa. *Journal of Plant Physiology* **163**: 445-451.

Liu, Y. G. and Chen, Y. 2007. High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Biotechniques* **43**: 649-656.

Liu, Y. J., Xiu, Z. H., Meeley, R. and Tan, B. C. 2013. Empty pericarp5 encodes a pentatricopeptide repeat protein that is required for nitochondrial RNA editing and seed development in maize. *The Plant Cell* **25**: 868-883.

Lu, Y., Li, C., Wang, H., Chen, H., Berg, H. and Xia, Y. 2011. AtPPR2, an *Arabidopsis* pentatricopeptide repeat protein, binds to plastid 23S rRNA and plays an important role in the first mitotic division during gametogenesis and in cell proliferation during embryogenesis. *The Plant Journal* **67**: 13-25.

Ma, Z. Dooner, H. K. 2004. A mutation in the nuclear-encoded plastid ribosomal protein S9 leads to early embryo lethality in maize. *The Plant Journal* **37**: 92-103.

Magnard, J. L., Heckel, T., Massonneau, A., Wisniewski, J. P., Cordelier, S., Lassagne, H., Perez, P., Dumas, C. and Rogowsky, P. M. 2004. Morphogenesis of maize embryos requires ZmPRPL35-1 encoding a plastid ribosomal protein. *Plant Physiology* **134**: 649-663.

Maier, R. M., Neckermann, K., Igloi, G. L. and Kössel, H. 1995. Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *Journal of Molecular Biology* **251**: 614-628.

Maréchal, A., Parent, J. S., Veronneau-Lafortune, F., Joyeux, A., Lang, B. F. and Brisson, N. 2009. Whirly proteins maintain plastid genome stability in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **106**: 14693-14698.

McCarty, D. R., Settles, A. M., Suzuki, M., Tan, B. C., Latshaw, S., Porch, T., Robin, K., Baier, J., Avigne, W., Lai, J., Messing, J., Koch, K. E. and Hannah, L. C. 2005. Steady-state transposon mutagenesis in inbred maize. *The Plant Journal* **44**: 52-61.

McElver, J., Tzafrir, I., Aux, G., Rogers, R., Ashby, C., Smith, K., Thomas, C., Schetter, A., Zhou, Q., Cushman, M. A., Tossberg, J., Nickle, T., Levin, J. Z., Law,

M., Meinke, D. and Patton, D. 2001. Insertional mutagenesis of genes required for seed development in *Arabidopsis thaliana*. *Genetics* **159**: 1751-1763.

Meinke, D., Muralla, R., Sweeney, C. and Dickerman, A. 2008. Identifying essential genes in *Arabidopsis thaliana*. *Trends in Plant Science* **13**: 483-491.

Myers, A. M., James, M. G., Lin, Q., Yi, G., Stinard, P. S., Hennen-Bierwagen, T. A. and Becraft, P. W. 2011. Maize opaque5 encodes monogalactosyldiacylglycerol synthase and specifically affects galactolipids necessary for amyloplast and chloroplast function. *Th Plant Cell* **23**: 2331-2347.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of Bioscience* and *Bioengineering* **104**: 34–41.

Neuhaus, H. E. and Emes, M. J. 2000. Nonphotosynthetic metabolism in plastids. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**: 111-140.

Ohlrogge, J. and Browse, J. 1995. Lipid biosynthesis. The Plant Cell **7**: 957-970.

Olinares, P. D., Kim, J., Davis, J. I. and van Wijk, K. J. 2011a. Subunit stoichiometry, evolution, and functional implications of an asymmetric plant plastidClpP/R protease complex in *Arabidopsis*. *The Plant Cell* **23**: 2348-2361.

Olsen, O. A. 2004. Nuclear endosperm development in cereals and *Arabidopsis thaliana*. *The Plant Cell* **16**: S214-227.

Ostheimer, G. J., Williams-Carrier, R., Belcher, S., Osborne, E., Gierke, J. and Barkan, A. 2003. Group II intron splicing factors derived by diversification of an ancient RNA-binding domain. *The EMBO Journal* **22**: 3919-3929.

Pittard, J., Camakaris, H. and Yang, J. 2005. The TyrR regulon. *Molecular Microbiology* 55: 12–26.

Prikryl, J., Watkins, K. P., Friso, G., van Wijk, K. J. and Barkan, A. 2008. A member of the Whirly family is a multifunctional RNA- and DNA-binding protein that is essential for chloroplast biogenesis. *Nucleic Acids Research* **36**: 5152-5165.

Romani, I., Tadini, L., Rossi, F., Masiero, S., Pribil, M., Jahns, P., Kater, M., Leister, D. and Pesaresi, P. 2012. Versatile roles of *Arabidopsis* plastid ribosomal proteins in plant growth and development. *The Plant Journal* **72**: 922-934.

Sasaki, Y. and Nagano, Y. 2004. Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. *Bioscience, Biotechnology, and Biochemistry* **68**: 1175-1184

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E. and Tabata, S. 1999. Complete structure of the chloroplast genome of *Arabidopsis thaliana*. *DNA Research* **6**: 283-290.

Sauer, M. and Friml, J. 2004. *In vitro* culture of *Arabidopsis* embryos within their ovules. The *Plant Journal* **40**:835-843.

Schmitz-Linneweber, C., Williams-Carrier, R. E., Williams-Voelker, P. M., Kroeger, T. S., Vichas, A. and Barkan, A. 2006. A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast rps12 pre-mRNA. *The Plant Cell* **18**: 2650-2663.

Schnable, P. S., Ware, D., Fulton, R. S., Stein, J. C., Wei, F., et al. 2009. The B73 maize genome: complexity, diversity, and dynamics. *Science* **326**: 1112-1115.

Schuller, D.J., Grant, G. A. and Banaszak, L. J. 1995. The allosteric ligand site in the V-max-type cooperative enzyme phosphoglycerate dehydrogenase. *Nature Structural & Molecular Biology* **2**: 69-76.

Schulte, W., Töpfer, R., Stracke, R., Schell, J. and Martini, N. 1997. Multi-functional acetyl-CoA carboxylase from *Brassica napus* is encoded by a multi-gene family: Indication for plastidic localization of at least one isoform. *Proceedings of the National Academy of Sciences* **94**: 3465–3470.

SeedGends: www.seedgenes.org/GeneList

Seo, M. and Koshiba, T. 2002. Complex regulation of ABA biosynthesis in plants. *Trends in Plant Science* **7**: 41-48.

Settles, A. M., Latshaw, S. and McCarty, D. R, 2004. Molecular analysis of high-copy insertion sites in maize. *Nucleic Acids Research* 32: e54.

Shen, H. B. and Chou, K. C. 2007. Nuc-PLoc: a new web-server for predicting protein subnuclear localization by fusing PseAA composition and PsePSSM. *Protein engineering design & selection* **20**: 561-567.

Shen, Y., Li, C., McCarty, D. R., Meeley, R. and Tan, B. C. 2013. Embryo defective12 encodes the plastid initiation factor 3 and is essential to embryogenesis in maize. *The Plant Journal* **74**: 792-804.

Shikanai, T., Shimizu, K., Ueda, K., Nishimura, Y., Kuroiwa, T. and Hashimoto, T. 2001. The chloroplast clpP gene, encoding a proteolytic subunit of ATP-dependent protease, is indispensable for chloroplast development in tobacco. *Plant Cell Physiology* **42**: 264–273.

Shintani, D., Roesler, K., Shorrosh, B., Savage, L. and Ohlrogge, J. 1997. Antisense expression and overexpression of biotin carboxylase in tobacco leaves. *Plant Physiology* **114**: 881-886.

Sjögren, L. L., Stanne, T. M., Zheng, B., Sutinen, S. and Clarke, A. K. 2006. Structural and functional insights into the chloroplast ATP-dependent Clp protease in *Arabidopsis*. *The Plant Cell* **18**: 2635-2649.

Small, I., Peeters, N., Legeai, F. and Lurin, C. 2004. Predotar: A tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics* **4**: 1581-1590.

Sosso, D., Canut, M., Gendrot, G., Dedieu, A., Chambrier, P., Barkan, A., Consonni, G., and Rogowsky, P. M. 2012. PPR8522 encodes a chloroplast-targeted pentatricopeptide repeat protein necessary for maize embryogenesis and vegetative development. *Journal of Experimental Botany* **6***3*: 5843-5857.

Springer, N. M., Ying, K., Fu, Y., Ji, T. M., Yeh, C. T., Jia, Y., Wu, W., Richmond, T., Kitzman, J., Rosenbaum, H., Iniguez, A. L., Barbazuk, W. B., Jeddeloh, J. A., Nettleton, D. and Schnable, PS. 2009. Maize Inbreds Exhibit High Levels of Copy Number Variation (CNV) and Presence/Absence Variation (PAV) in Genome Content. *Plos Genetics* **5**: e1000734.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**: 31-43.

Strable, J. and Scanlon, M. J. 2009. Maize *(Zea mays):* a model organism for basic and applied research in plant. *Cold Spring Harbor Protocals* **4**: doi:10.1101/pdb.emo132.

Stupar, R. M. and Springer, N. M. 2006. Cis-transcriptional variation in maize inbred lines B73 and Mo17 leads to additive expression patterns in the F1 hybrid. *Genetics* **173**: 2199-2210.

Sugiura, M. 1992. The chloroplast genome. *Plant Molecular Biology* **19**: 149-168.

Sung, T. Y., Chung, T. Y., Hsu, C. P. and Hsieh, M. H. 2011. The ACR11 encodes a novel type of chloroplastic ACT domain repeat protein that is coordinately expressed with GLN2 in Arabidopsis. *BMC Plant Biology* **11**:118.

Sylvester, A. W. and Ruzin, S. 1994. Light microscopy I: Dissection and microtechnique and II: Image analysis. In M Freeling, and V Walbot (eds). *The Maize Handbook*. New York: Springer-Verlag. pp. 83-107.

Tan, B. C., Chen, Z., Shen, Y., Zhang, Y., Lai, J. and Sun, S. S. M. 2011. Identification of an active new mutator transposable element in maize. *Genes, Genomes, Genetics* **1**: 293-302.

Tzafrir, I., Pena-Muralla, R., Dickerman, A., Berg, M., Rogers, R., Hutchens, S., Sweeney, T. C., McElver, J., Aux, G., Patton, D. and Meinke, D. 2004. Identification of genes required for embryo development in *Arabidopsis*. *Plant Physiology* **135**: 1206-1220.

Wakasugi, T., Tsudzuki, T. and Sugiura, M. 2001. The genomics of land plant chloroplasts: Gene content and alteration of genomic information by RNA editing. *Photosynthesis Research* **70**: 107-118.

Walsh, J., Waters, C. A. and Freeling, M. 1998. The maize gene *liguleless2* encodes a basic leucine zipper protein involved in the establishment of the leaf blade–sheath boundary. *Genes & Development* **12**: 208–218.

Williams, P. M. and Barkan, A. 2003. A chloroplast-localized PPR protein required for plastid ribosome accumulation. *The Plant Journal* **36**: 675-686.

Woodson, J. D., and Chory, J. 2008. Coordination of gene expression between organellar and nuclear genomes. *Nature Reviews Genetics* **9**: 383-395.

Xiong, J. Y., Lai, C. X., Qu, Z., Yang, X. Y., Qin, X. H. and Liu G. Q. 2009. Recruitment of AtWHY1 and AtWHY3 by a distal element upstream of the kinesin gene AtKP1 to mediate transcriptional repression. *Plant Molecular Biology* **71**: 437-449.

Yamaguchi, S. and Kamiya, Y. 2000. Gibberellin biosynthesis: its regulation by endogenous and environmental signals. *Plant Cell Physiology 41*: 251-257.

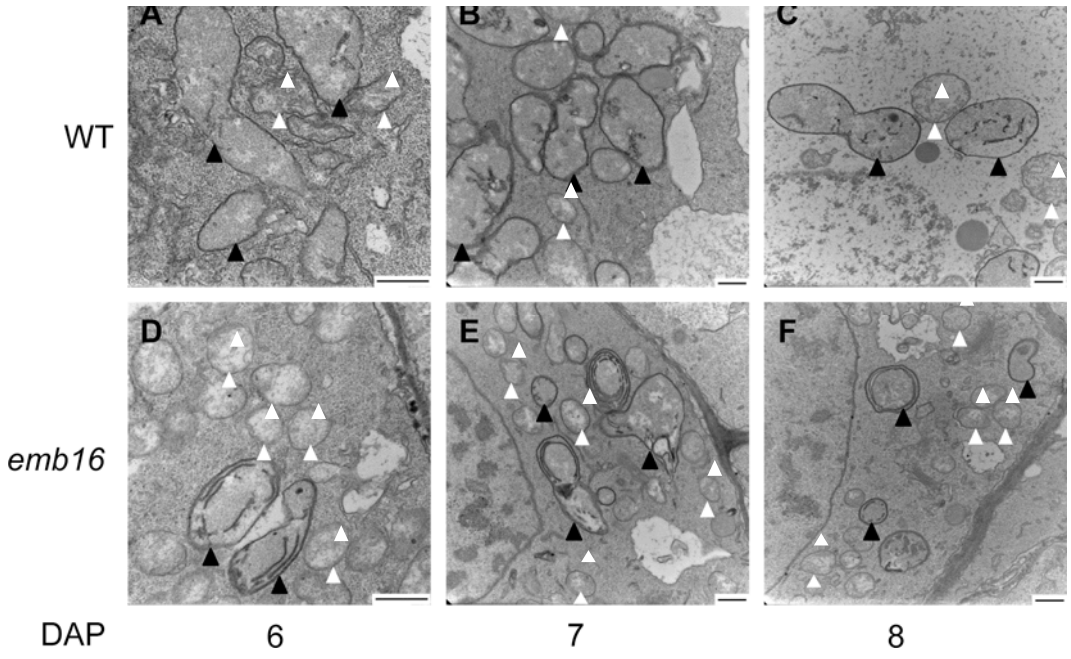
Yoo, H. H., Kwon, C., Lee, M. M. and Chung, I. K. 2007. Single-stranded DNA binding factor AtWHY1 modulates telomere length homeostasis in *Arabidopsis*. *The Plant Journal* **49**: 442-451.

Zhang, H. B., Zhao, X., Ding, X., Paterson, A. H. and Wing, R. A. 1995. Preparation of megabase-size DNA from plant nuclei. *The Plant Journal* **7**: 175-184.

Zheng, B., MacDonald, T. M., Sutinen, S., Hurry, V. and Clarke, A. K. 2006. A nuclear-encoded ClpP subunit of the chloroplast ATP-dependent Clp protease is essential for early development in Arabidopsis thaliana. *Planta* **224**: 1103-1115

Appendix

Appendix 1.1 Development of proplastid and mitochondrion in *emb16* mutant.



Appendix 1.1 Development of proplastid and mitochondrion in *emb16* mutant. The WT and *emb16* mutant kernels from segregating ears were sectioned from 6 to 14 DAP. At 6 DAP, *emb16* embryo is distinguished from WT by the size and structure of embryo proper using stereomicroscopy and confirmed by the endosperm genotyping. The ultrastructural observation of embryo cells for *emb16* mutant is from the embryo proper cells and for WT is from embryo proper cells (6 DAP) or shoot meristem cells (7 and 8 DAP). Embryo proper cells are different from suspensor cells, which contain more starch granules and vacuoles. Similar cell contents were observed in cells of shoot meristem, leaf primordia, and coleoptile in the WT embryo. Empty arrow heads point to mitochondria, and filled arrow heads point to proplastids. Scale bars =0.5µm.

Appendix 2.1 Publications

1. Ya-Feng Zhang, Ming-Ming Hou, Bao-Cai Tan. 2013. The Requirement of WHIRLY1 for Embryogenesis Is Dependent on Genetic Background in Maize. *PLoS One* **8**: e67369.
2. Bao-Cai Tan, Zongliang Chen, Yun Shen, Yafeng Zhang, Jinsheng Lai, Samuel S. M. Sun. 2011. Identification of an Active New *Mutator* Transposable Element in Maize. *G3 (Bethesda)* **1**: 293–302.