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Functional Characterization Of The Plant 15-Cis-Zeta-Carotene Isomerase Z-Iso

Jesus Alonso Beltran Zambrano
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FUNCTIONAL CHARACTERIZATION OF THE PLANT 15-CIS-ZETA-CAROTENE ISOMERASE (Z-ISO)

by

JESÚS ALONSO BELTRÁN ZAMBRANO

A dissertation submitted to the Graduate Faculty of Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

2015
This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the Doctor of Philosophy.

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Abstract

FUNCTIONAL CHARACTERIZATION OF THE PLANT 15-CIS-ZETA-CAROTENE ISOMERASE Z-ISO

by

Jesús Alonso Beltrán Zambrano

Advisor: Professor Eleanore T. Wurtzel

Vitamin A deficiency is a widespread health issue in the tropics. To solve this issue, efforts are underway to increase provitamin A carotenoids such as β-carotene in staple crops which can be achieved by breeding, metabolic engineering or a combination of both approaches. However, rational strategies to improve carotenoid content in crops require sufficient knowledge of pathway regulation. Therefore, to better understand how plants synthesize provitamin A and to guide metabolic engineering strategies in crops such as maize, the functional characterization of the new ζ-carotene isomerase (Z-ISO) is of significant importance.

Z-ISO was recently discovered in maize and Arabidopsis (Chen et al., 2010). This new enzyme is a 15-cis-ζ-carotene isomerase present in all plants, diatoms and algae. Z-ISO is required in both green and non-green tissues including roots and the seed endosperm which is target for provitamin A biofortification. In this dissertation, to gain a better understanding of the role of Z-ISO in the isomerization of 15-cis-ζ-carotene, the Z-ISO polypeptide was biochemically characterized using extensive spectroscopy and its function was examined by
developing an *in vitro* enzymatic assay and by an *in vivo* complementation system in *Escherichia coli*.

Bioinformatic tools modeled Z-ISO as an integral membrane and heme or non-heme iron binding protein. Therefore, using the Z-ISO polypeptide sequence, we selected conserved putative residue ligands for heme and non-heme iron and mutagenized them to Alanine. These Z-ISO mutant versions were tested for enzymatic function using an *E. coli* complementation system. These experiments showed that from all the conserved histidines present in Z-ISO, two (H150 and H266) as well as one aspartic acid residue (D294), were essential for isomerase activity. The only cysteine (C263) residue present in Z-ISO was not required for activity. These results are in good agreement with the predicted Z-ISO model where the locations for H150 and H266 are consistent with the coordination of a common factor.

Maize Z-ISO was also over-expressed and purified as a TEV protease-cleavable, maltose binding protein (MBP) fusion (MBP::Z-ISO). An *in vitro* assay utilizing substrate containing liposomes was developed to test Z-ISO activity. The conversion of the substrate 9,15,9'-tri-cis-ζ-carotene into the product 9,9'-di-cis-ζ-carotene by Z-ISO proceeded under reducing conditions but not under oxidizing conditions.

MBP::Z-ISO purified protein was also tested for the presence of metals. Using inductively coupled plasma optical emission spectrometry (ICP-OES) iron was detected but there were no significant levels of Ca, Cu, Mg, Mn, Mo, or Zn. Therefore, we concluded that Z-ISO is
a metalloprotein. *E. coli* culture pellets expressing MBP::Z-ISO are brown consistent with the presence of iron or heme.

The presence of heme in purified MBP::Z-ISO was evaluated using heme staining and pyridine hemochrome assays. Heme staining of protein separated in SDS-PAGE gels revealed that MBP::Z-ISO and Z-ISO contain heme while MBP alone does not. Moreover, hemochrome assays showed the presence of heme $b$ in Z-ISO. The UV-visible absorption spectrum of the intact, as isolated Z-ISO confirmed the presence of heme $b$ in the oxidized, ferric Fe (III) state. Also, the spectrum of reduced Z-ISO is similar to that of cytochromes containing heme $b$ with two axial histidine ligands and the reduced heme $b$ binds carbon monoxide (CO).

Z-ISO was also characterized using electron paramagnetic resonance (EPR). An X-band spectrum of the MBP::Z-ISO fusion detected high-spin ferric heme (e.g. heme $b$ with a single histidine ligand), multiple low-spin heme species, and a non-heme iron center. EPR also indicated the presence of two low-spin heme species and that one of these hemes might have a bis-histidine coordination and the other might have a histidine-cysteine axial coordination. Reduced Z-ISO also binds NO which is consistent with the binding of CO.

To gain more details on the heme cofactor, the same sample used in the EPR experiments was used to characterize the heme iron in MBP::Z-ISO using magnetic circular dichroism (MCD) which detects only heme iron. MCD characterization showed that Z-ISO has two ligand pairs (His/His and His/Cys), a result that is consistent with the EPR results. MCD experiments also
showed a redox-dependent change in ligand (Cys↔His) coordination of a low-spin heme \(b\).

Our data also suggested the existence of a high-spin, 5-coordinate, His-ligated heme which was detected by EPR and as a minor species by MCD. We hypothesized that substrate could bind to this intermediate or that substrate displaces an axial ligand to coordinate with the heme iron. MCD, EPR, and UV-Vis analysis showed that exogenous ligands bind the Fe(II) state but it was not known whether an exogenous ligand can displace an axial ligand when the heme is in the Fe(III) state. To test this possibility we utilized addition of cyanide (CN\(^-\)), which binds preferably to the ferric rather than the ferrous heme. CN\(^-\) was added to both the as isolated (oxidized) Fe(III) ferric enzyme and to the Fe(II) (dithionite-reduced) ferrous enzyme. UV-Vis absorption spectra indicated CN\(^-\) binding to the ferric Fe(III) heme which suggest that Z-ISO can also bind exogenous ligands in the oxidized form. Taken together our data suggest that heme is essential for Z-ISO activity. The presence of heme and its requirement for Z-ISO activity are surprising since heme is not often described in isomerization reactions. Based on our results, we propose a mechanism for Z-ISO function.
I am greatly thankful to my advisor, Dr. Eleanore T. Wurtzel, for her valuable guidance, encouragement and support throughout my doctoral studies. Her continuous advice was essential for the successful completion of this doctoral work. I am also grateful to her for giving me the opportunity to join her laboratory.

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I extend special thanks to our various collaborators: Dr. Brian Kloss (New York Structural Biology Center, NYSBC), Dr. Wayne Hendrickson (Columbia University and NSBC) and Dr. James Love (NYSBC) for their help with protein expression and purification, Dr. Jonathan Hosler (University of Mississippi) for sharing his expertise in heme proteins and his help with spectroscopic studies, Dr. Aimin Liu and Jiafeng Geng (Georgia State University) for their help with EPR studies, Dr. John H. Dawson, Dr. Masanori Sono and Dr. Anuja Modi (University of South Carolina) for their help with MCD studies.

I also wish to acknowledge all the members in the Wurtzel’s laboratory past and present for their help: Dr. Rena Quinlan, Dr. Yu Chen, Dr. Oren Tzafadia, Dr. Louis Bradbury, Dr. Maria Shumskaya, Dr. Regina Monaco, Xiaoling Yao and Dr. Charles Dwamena.
Finally, I thank all the members of the Biology Department at Lehman College and all the members at the CUNY GC Biology Ph.D program. On a personal note, I extend thanks to my family and friends for their continuous support.
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<tr>
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<td>carotenoid isomerase</td>
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<td>EPR</td>
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<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NO</td>
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CHAPTER 1

Introduction

1.1 Carotenoids: background and significance

Carotenoids are a large class of structurally diverse isoprenoids (Britton et al., 2004) synthesized in photosynthetic organisms, some bacteria, fungi, and arthropods (Moise et al., 2014). By virtue of their absorption and transfer of photonic energy, carotenoids protect plants against photooxidative stress, serve as light harvesters in photosynthesis (Havaux and Niyogi, 1999; Niyogi, 2000; Johnson et al., 2007) and confer color to non-green tissues like flowers, seeds and fruits. In plants, carotenoids are also important precursors of hormones (Schwartz et al., 1997; Gomez-Roldan et al., 2008; Umehara et al., 2008; Messing et al., 2010) essential for signaling and developmental responses (Messing et al., 2010; Walter et al., 2010).

The distinctive yellow, orange, and red pigments of carotenoids are exploited by plants to attract pollinators (Kato et al., 2004). In humans and other mammals these pigments are essential components of the diet having anti-oxidant and provitamin A activities (van den Berg et al., 2000; von Lintig, 2010).

Because humans lack the enzymatic machinery for the generation of vitamin A de novo, the primary source of β-carotene and other provitamin A carotenoids is the diet. Unfortunately, the cereal crops which are the main source of calories and nutrition globally (Welch and Graham, 2004), are in most cases deprived in required levels of carotenoids, especially provitamin A (Graham, 1997; Harjes et al., 2008; Wurtzel et al., 2012). Therefore, increasing the level of
provitamin A carotenoids in plant food staples using breeding or metabolic engineering represents an alternative approach to solve malnutrition problems associated with vitamin A deficiency (Wurtzel et al., 2012).

1.2 Chemical structure of carotenoids

Carotenoids are C\textsubscript{40} terpenoids formed by the condensation of two molecules of C\textsubscript{20} geranylgeranyl diphosphate. A distinctive feature of carotenoids is the polyene skeleton consisting in a long array of alternating conjugated double and single bonds resulting in a chromophore. Delocalized \(\pi\)-electrons along the polyene chain are responsible for light absorbing properties, shape and reactivity of carotenoids. The polyene chain of carotenoids undergoes geometric isomerization of the double bonds which generates either E-configurations (\textit{trans}-configurations) or Z-configurations (\textit{cis}-configurations) (Britton et al., 1995).

In general, there are two distinctive groups of carotenoids; the xantophylls (ie., lutein), which result from oxygenation, and the carotenes (ie., \(\beta\)-carotene), which are non-oxygenated, linear or having cyclic hydrocarbons at one or both ends of the molecule. Carotenoids are precursors to a growing variety of cleavage products such abscisic acid (ABA) and strigolactones (Walter et al., 2010; Cuttriss et al., 2011; Moise et al., 2014).
1.3 Carotenoid pathway localization

Carotenoids are synthesized in the membranes of different plant plastids such as chloroplasts, amyloplasts, and chromoplasts. Carotenogenic enzymes are encoded by nuclear genes and imported into plastids where their transit peptides are removed. These enzymes are either peripherally or integrally bound to the thylakoids or localized in the stroma. However, the exact three-dimensional structure of the pathway and metabolon organization remains underinvestigated (Shumskaya and Wurtzel, 2013).

In maize, immunoassay experiments showed that PSY1 localizes to envelope membranes of amyloplasts (Li et al., 2008b). Using transient expression of GFP fusions in protoplasts Zm-PSY1 localizes to the stroma while Zm-PSY2 and Zm-PSY3 localize to plastoglobuli (Shumskaya et al., 2012). Interestingly, the localization of PSY is altered by isozyme, allelic variation, and activity in protoplasts (Shumskaya et al., 2012). In Arabidopsis, proteomics experiments showed that PDS, ZDS, CRTISO, the P450 β- and ε-ring hydroxylases (CYP97A and CYP97C), as well as β-cyclase (LCYB) and zeaxanthin epoxidase (ZEP) localize to envelope membranes of chloroplasts. PDS and ZEP however were also found in thylakoid membranes as well as violaxanthin de-epoxidase (VDE) (Joyard et al., 2009).

1.4 Carotenoid biosynthesis

The carotenoid biosynthetic pathway begins with the formation of the colorless compound phytoene catalyzed by phytoene synthase (PSY) (reviewed in Cuttriss, 2011). The subsequent steps of the pathway towards formation of colored carotenes (all trans lycopene) involve two desaturation steps performed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS)
(Beyer et al., 1989; Hugueney et al., 1992; Bartley et al., 1999; Matthews et al., 2003; Breitenbach and Sandmann, 2005) and two isomerizations performed by the new component Z-ISO (ζ-carotene isomerase) and CRTISO (cis-trans isomerase) (Isaacson et al., 2004). Each desaturase (PDS and ZDS) introduces two double bonds for a total of 4 double bonds.

In summary, the sequence of desaturation and isomerization reactions proceeds in the order PDS → Z-ISO → ZDS → CRTISO (Fig.1.1), although more details need to be understood about the mechanism of reaction employed by these enzymes (Moise et al., 2014). Using phytoene as substrate, PDS generates 9,15,9′-tri-cis-ζ-carotene. The 15-cis bond is then isomerized by Z-ISO, which generates di-cis-ζ-carotene, the substrate for ZDS. Once 7,9,9′,7′-tetra-cis-Lycopene is generated by ZDS, all-trans-lycopene is then generated by CRTISO. In contrast to algae and plants, this complete multi-enzymatic process is performed by the single enzyme CRTI in bacteria (Linden et al., 1991). Plant CRTISO and bacterial CrtI share ancestry and they both bind flavin adenine dinucleotide (FAD) as the cofactor underlying the isomerization reactions (Yu et al., 2011). In contrast, Z-ISO lacks a predicted sequence for FAD binding.

In plants, plastoquinones serve as acceptors of the electrons released by the desaturation reactions (Mayer et al., 1990; Norris et al., 1995). In photosynthetic tissue the regeneration of plastoquinones occurs via photosynthetic electron transfer while in non-green tissue it occurs via an alternative oxidase (Sandmann, 2009).

A well-defined major branch point in the carotenoid biosynthesis pathway is represented by the cyclization of the linear compound lycopene. Two enzymes, lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) catalyze the formation of ε- and β-rings, respectively. LCYB
produces β-carotene by adding rings at both ends of lycopene while the combined activities of LCYB and LCYE enzymes add one β-ring and one ε-ring to the ends of lycopene, respectively (Quinlan et al., 2012).

Hydroxylation of the β- and ε-rings of the β- and α-carotene precursors leads to biosynthesis of non-provitamin A xanthophylls such as lutein and zeaxanthin. These compounds are produced by the action of carotenoid β- and ε-hydroxylases, by adding hydroxyl groups to the third carbon of carotene β- and ε-rings, respectively. β-ring hydroxylations may involve either cytochrome P450-type (CYP97A) β- or diiron-type (HYDB) hydroxylases. CYP97C is a cytochrome P450 ε-ring hydroxylase identified in Arabidopsis (Tian et al., 2004). The diiron-HYDs have been characterized from different organisms, including bacterial, algal, and plants species (Sun et al., 1996; Bouvier et al., 1998; Tian et al., 2003; Tian and DellaPenna, 2004; Vallabhaneni et al., 2009). CYP97A is a β-ring hydroxylase (Fiore et al., 2006; Kim and DellaPenna, 2006), and both OsCYP97A and OsCYP97C enzymes were functionally demonstrated in E. coli complementation systems (Quinlan et al., 2007). Recently, it was shown that efficient formation of lutein, which involves carotenoid hydroxylation, requires the coexpression of CYP97A and CYP97C enzymes (Quinlan et al., 2012). In vivo and in vitro, these enzymes physically interact which explains carotenoid pathway flux in plants (Quinlan et al., 2012).

Further addition of oxygen mediated by the enzyme zeaxanthin epoxidase (ZEP) converts zeaxanthin to epoxy-xanthophylls (i.e. antheraxanthin and violaxanthin). This reaction is reversible under high–light stress in a process referred as to the xanthophyll cycle (Jahns and Holzwarth, 2012).
1.5 Carotenoid cleavage products: apocarotenoids

Carotenoids serve as precursors for apocarotenoids which are important signaling molecules that function in different biological processes. Apocarotenoids with biological importance include vitamin A retinoids (retinal, retinoic acid, and retinol) and the plant hormone abscisic (ABA). These molecules are tailored from carotenoids such as β-carotene and zeaxanthin via oxidative cleavage by the action of carotenoid cleavage dioxygenases (CCDs) (Auldridge et al., 2006; Schmidt et al., 2006).

As a distinctive signature, all the CCD family members contain a conserved carboxyl terminus peptide sequence (Auldridge et al., 2006). They all are iron binding proteins and are distinguished based on their particular cleavage sites (Floss and Walter, 2009). The maize Vp14 gene product is a nine-cis-epoxy dioxygenase (NCED) involved in ABA biosynthesis (Schwartz et al., 1997; Tan et al., 1997). Using 9-cis-epoxycarotenoids (i.e neoxanthin and violaxanthin) as substrates, NCEDs can specifically cleave their 11, 12 double-bond positions. Other CCDs acting on all-trans carotenoid substrates asymmetrically cleave the 9,10 bond or symmetrically cleave the 9,10 (9′,10′) bonds, or 7,8 (7′,8′) and 5,6 bonds (Marasco et al., 2006).

Most of the CCD enzymes localize to the plastid but AtCCD1 is cytosolic producing C_{13} and C_{14} apocarotenoids (Auldridge et al., 2006; Auldridge et al., 2006). In maize, the expression of CCD1 alleles of the white cap 1 locus (wcl) correlates with reduced carotenoid content in the endosperm (Vallabhaneni et al., 2010). Additionally, high CCD1 copy number in a maize inbred lines correlates with low carotenoid content in the endosperm (Vallabhaneni et al., 2010).
Strigolactones are also a group of carotenoid-derived molecules acting as hormones in plants. These molecules stimulate germination of parasitic plants and promote hyphal branching of mycorrhizal fungi (Akiyama et al., 2005; Matusova et al., 2005). In roots, strigolactones are also involved in branching (Floss and Walter, 2009).

Biosynthesis of strigolactones is mediated by the enzymes CCD7 and CCD8. These enzymes mediate two-step consecutive cleavage of β-carotene. The product (a C18 intermediate) is predicted to serve as precursor for strigolactones. The precursor undergoes modifications before being transported to the shoot where it regulates branching (Floss and Walter, 2009).

Recently, the pathway from β-carotene to the strigolactone-like hormone, carlactone, was elucidated using in vitro experiments (Alder et al., 2012). This study showed that D27 is an iron-binding β-carotene isomerase, which catalyzes the isomerization of the 9,10 double bond in all-trans-β-carotene to generate 9-cis-β-carotene. The latter compound is then cleaved by CCD7 at the 9′,10′ position to produce the aldehyde 9-cis-β-apo-10′-carotenal. Then, 9-cis-β-apo-10′-carotenal gains three oxygens by the action of CCD8 to produce carlactone. Whether D27 is a heme binding isomerase remains to be investigated.

1.6 15-cis-ζ-carotene isomerase (Z-ISO)

As mentioned before, Z-ISO is one of the most recent components of the core carotenoid biosynthetic pathway. As this dissertation focuses on Z-ISO, the available data related to this enzyme is described in more detail in this section.
1.6.1 Prediction of the existence of Z-ISO

The existence of the missing enzyme, Z-ISO (15- cis-ζ-carotene isomerase), was predicted based on the carotenoid profile of the maize mutant pale yellow9 (y9) (Li et al., 2007). In this mutant, recessive alleles accumulated 9,15,9′-tri-cis-ζ-carotene in dark tissues. In the presence of light the 15-15′ cis double bond in 9,15,9′-tri-cis-ζ-carotene of y9 photosynthetic tissue is photoisomerized therefore releasing the pathway block enabling plants to become “green”. Nevertheless, comparisons on the carotenoid content between Z-ISO mutant and wild type leaves led to the conclusion that light only partially compensates for the absence of Z-ISO (Li et al., 2007; Chen et al., 2010).

1.6.2 Isolation of the Z-ISO gene

Maize mutant lines led to the discovery of the missing enzyme, Z-ISO (15- cis-ζ-carotene isomerase). The y9 allele from maize was identified as encoding a factor required for isomerase activity which functions in dark-grown tissues in catalyzing the cis- to trans-conversion of the 15-15′ cis double bond in 9,15,9′-tri-cis-ζ-carotene, the desaturation product of PDS, to generate 9,9′-di-cis-ζ-carotene, the suitable geometrical isomer substrate for ζ-carotene desaturase (ZDS) (Li et al., 2007). To accelerate Z-ISO gene isolation, a screening strategy to identify Arabidopsis mutants accumulating 9,15,9′-tri-cis-ζ-carotene was initiated. The Z-ISO gene was then identified using map-based cloning. The mutated gene was At1g10830 (Chen et al., 2010). Using the Arabidopsis sequence as a query it was possible to identify the maize Z-ISO gene (BT036679) encoded by the y9 locus (Chen et al., 2010).
The Z-ISO gene is present throughout oxygenic autotrophs as a single copy gene (Chen et al., 2010). Unexpectedly, Z-ISO doesn’t share ancestry with CRTISO or with other enzymes of the pathway like PDS or ZDS which are very similar at the amino acid sequence level. The Z-ISO protein is related to NnrU, a protein required for bacterial denitrification (Bartnikas et al., 1997). Phylogenetic analysis using Z-ISO sequences from higher and lower plants, algae, diatoms, and cyanobacteria suggested that Z-ISO evolved from a common progenitor of NnrU (Chen et al., 2010).

### 1.6.3 Other studies on Z-ISO

Most of the available knowledge on Z-ISO comes from studies done in maize and Arabidopsis (Li et al., 2007; Chen et al., 2010). Although Z-ISO is a relatively new enzyme there are few new studies that confirm its role in plant carotenogenesis. In tomato, Z-ISO is highly expressed in fruits and silencing its mRNA levels resulted in a pale-red phenotype due to reduction of lycopene and increase of phytoene, phytofluene and, as expected, ζ-carotene (Fantini et al., 2013). In an independent study, also done in tomato, expression analysis suggested that the Z-ISO gene is positively and directly regulated by the tomato MADS box transcription factor RIPENING INHIBITOR (RIN) (Fujisawa et al., 2013). Moreover, the expression of Z-ISO was identified as a potential rate-limiting step for carotenoid biosynthesis in apple fruits since Z-ISO transcript levels correlated with fruit skin and flesh carotenoid content (Ampomah-Dwamena et al., 2012). Therefore, Z-ISO represents a potential to improve nutritional value in various crops.

Despite the importance of Z-ISO, its mechanism of function was unknown. Homology modeling using bioinformatics tools modeled the residues of Z-ISO onto the diheme cytochrome b subunit.
of quinol; fumarate oxidoreductase, an integral membrane protein (Wurtzel, unpub). Therefore, one of the aims of this dissertation was to test whether Z-ISO binds heme as the cofactor used in \( \zeta \)-carotene isomerization (Chapter 2).

### 1.7 Heme binding proteins

Metalloproteins account for about half of the proteins in nature (Lu et al., 2009). Heme proteins are metalloproteins that contain iron porphyrin as a cofactor. Porphyrin is a large heterocyclic organic ring made up of four pyrrolic groups which are joined together by methane bridges. Iron is coordinated by the four nitrogens of the heme prophyrin ring and above and below this plane by 1 or 2 amino acid ligands within the protein (e.g. a high spin 5-coordinate heme or a low-spin 6-coordinate heme), respectively. This cofactor is used by heme proteins to catalyze a diverse array of reactions ranging from electron transfer (e.g. cytochrome c, cyt c and cytochrome b5, cyt b5), catalysis (e.g. cytochrome P450, CYP), oxygen binding and transport (e.g. myoglobin, Mb and hemoglobin, Hb) and signaling (e.g. CO sensor CooA) (Lu et al., 2001; Lu et al., 2009).

In heme proteins, the type of reaction is controlled mainly by the proximal axial ligand, the residues surrounding it, the architecture of the distal site, and the type of heme (Lu et al., 2001). It is well established that the axial ligands play important roles in modulation of structure and function of heme proteins, such as electronic structure, redox potentials, spin states, electron-transfer rates, and catalytic properties (Lu et al., 2001). Several amino acids can serve as axial or proximal ligands to heme proteins. Bis-His coordination, present in cyt b5 and His-Met, present in cyt c, are the most common ligation forms of the cytochromes (Lu et al., 2001). In enzymes and sensors the heme is mainly five coordinate with an open site for CO or O\(_2\) binding. In those
proteins, the most common axial ligand is histidine, while tyrosine and cysteine are found in a few classes of enzymes such catalase, cyt P450 and chloroperoxidase (CPO) (Lu et al., 2001).

1.8 Objectives
In plants, carotenoids are essential for development and survival. In humans, cleavage of the carotenoid β-carotene forms vitamin A, a key component of the human diet (von Lintig, 2010). Nonprovitamin A carotenoids are also beneficial in human health. In the developing world, the consumption of carotenoid-poor crops is associated with vitamin A deficiency which represents a serious global health problem (Sommer and Davidson, 2002). The endosperm tissue of staple crops such as maize is low in provitamin A content and therefore a target for provitamin A improvement (Wurtzel et al., 2012).

Attempts to enhance provitamin A content in the endosperm of crops such maize and rice require elucidation of the mechanisms underlying plant carotenoid biosynthesis and accumulation of provitamin A. Although the carotenoid biosynthetic pathway has been extensively studied, Z-ISO was only recently discovered (Chen et al., 2010). Therefore, the objective here was to elucidate the mechanism and regulation of Z-ISO isomerization. In order to gain a better understanding of this new component, the work described herein examined the features and activity of maize Z-ISO at the molecular and biochemical level.

Since plants with insufficient Z-ISO grow poorly under the stress of fluctuating temperature (Robertson, 1975; Janick-Buckner et al., 2001) studying the function of Z-ISO will be also important to understand how plants adapt to environmental stress.
1.8.1 Hypotheses

The central hypothesis is that cis to trans isomerization of the 15-15’ C=C bond in 9,15,9’-cis-ζ-carotene is mediated by Z-ISO through a metal cofactor.

1.8.2 Specific aims

(1). Overexpress Z-ISO as a fusion protein to facilitate its characterization.

Z-ISO was successfully expressed in E. coli which allowed the identification and characterization of a heme b cofactor (Chapter 2).

(2). Test whether Z-ISO functions independently as a bona fide enzyme.

An in vitro enzymatic assay using substrate-containing liposomes was developed to demonstrate that Z-ISO is an enzyme and to evaluate its functional requirements with respect to oxidation state of the heme iron (Chapter 2).

(3). Identify the cofactor and ligands in Z-ISO.

Z-ISO mutant versions were evaluated via E. coli functional complementation and spectroscopy to identify potential ligands required for heme ligation (Chapter 2 and 3).

The following appendices contain supplementary information and experimental protocols related to this research and to an earlier co-authored paper:


Appendix 3. Separation of ζ-carotene isomers by HPLC.
Appendix 4. Generation of antibodies against Z-ISO.

Appendix 5. A co-authored publication related to this dissertation.

Appendix 6. Plasmids used in this study and laboratory clone sheets.
Figure 1.1. Carotenoid biosynthesis until lycopene. PSY, Phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase (revised from (Moise et al., 2014))
CHAPTER 2

Redox control of carotenoid biosynthesis through a new heme isomerase

2.1 Abstract

Plants synthesize an array of carotenoid compounds that are essential for plant development and survival. These metabolites also serve as essential nutrients for human health. The biosynthetic pathway leading to all plant carotenoids includes the recently discovered 15-cis-ζ-carotene isomerase (Z-ISO). It was not certain whether isomerization was achieved by Z-ISO alone or in combination with other enzymes. Here we show that Z-ISO is a *bona fide* isomerase that independently catalyzes the *cis*-to-*trans* isomerization of the 15-15’ C=C bond in 9,15,9’-*cis-*ζ-carotene. Z-ISO was found to be a heme *b*-containing integral membrane protein. We discovered that isomerization is regulated by heme redox state through ligand rearrangement. Heme *b*-dependent isomerization of a specific C=C bond of a large, hydrophobic compound in a membrane is unprecedented. Z-ISO represents a new type of isomerase utilizing ferrous heme with redox-regulated ligand-switching necessary for isomerization. Thus, Z-ISO potentially utilizes a novel chemical mechanism within a membrane environment.

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1 This chapter is being prepared for journal submission: Redox control of carotenoid biosynthesis through a novel heme isomerase (2014). Jesús Beltrán, Brian Kloss, Jonathan P. Hosler, Jiafeng Geng, Aimin Liu, Anuja Modi, John H. Dawson, Masanori Sono, Maria Shumskaya, Charles Ampomah-Dwamena, James D. Love and Eleanore T. Wurtzel.
2.2 Introduction

Carotenoids constitute a large class of isoprenoids (Britton, 1985) synthesized by all photosynthetic organisms, some bacteria, fungi, and arthropods (Cuttriss, 2011; Cobbs et al., 2013; Moise et al., 2014). In humans, cleavage of the carotenoid ζ-carotene forms vitamin A (von Lintig, 2010; von Lintig, 2011). Nonprovitamin A carotenoids are also beneficial in human health (Giovannucci et al., 1995; Kohlmeier et al., 1997; Sommerburg et al., 1998; Fraser and Bramley, 2004). Carotenoids serve diverse roles in plants including photosynthetic light-harvesting and photoprotection (Dall'Osto et al., 2007), preventing lipid peroxidation for stress tolerance (Davison et al., 2002; Havaux et al., 2007; Johnson et al., 2007), and serving as precursors of hormones that signal stress and developmental responses (Li et al., 2008a; Messing et al., 2010; Walter et al., 2010). The plant carotenoid biosynthetic pathway is mediated by nuclear-encoded enzymes localized to chloroplasts or other plastids (Wurtzel et al., 2012; Moise et al., 2014) and organized within a multi-enzyme complex (Shumskaya and Wurtzel, 2013). Genes encoding the biosynthetic pathway are transcriptionally regulated (Meier et al., 2011; Ruiz-Sola and Rodriguez-Concepcion, 2012; Wurtzel et al., 2012) and the pathway is also post-translationally responsive to various signals such as stress. For example, nitric oxide (NO) produced in plastids inhibits carotenogenesis (Gas et al., 2009; Zhao et al., 2009; Chang et al., 2013). However, it is unknown how NO interferes with carotenogenesis nor whether any of the biosynthetic enzymes are direct NO targets. The carotenoid biosynthetic reactions also require an electron transfer chain and plastoquinones to channel electrons/protons produced during desaturation steps mediated by phytoene desaturase (PDS).
and ζ-carotene desaturase (ZDS). PDS produces 9,15,9'-tri-cis-ζ-carotene, which must be isomerized at the 15-15’ cis C=C bond to form 9,9'-di-cis-ζ-carotene, the substrate for a second desaturase, ZDS (Fig. 1). Isomerization of the double bond can be mediated by light. However, we hypothesized that an unknown isomerase must be required, since carotenogenesis occurs in “dark” tissues such as roots, etiolated leaves, and endosperm (Li et al., 2007). We discovered this isomerase and named it 15-cis-ζ-carotene isomerase (Z-ISO) (Chen et al., 2010). Without Z-ISO, carotenoid biosynthesis is blocked and 9,15,9'-tri-cis-ζ-carotene accumulates (Chen et al., 2010). Thus, Z-ISO is a gatekeeper of carotenoid pathway flux, especially in tissues without light exposure, such as the endosperm tissue targeted for improvement of provitamin A carotenoids (Li et al., 2007; Harjes et al., 2008; Chen et al., 2010; Wurtzel et al., 2012). Plants with insufficient Z-ISO also grow poorly under the stress of fluctuating temperature (Robertson, 1975; Janick-Buckner et al., 2001). Since climatic variations alter the need for photosynthetic and nonphotosynthetic carotenoids, Z-ISO facilitates plant adaptation to environmental stress, a major factor affecting crop yield. Thus, Z-ISO is essential for maximizing plant fitness in response to environmental changes and for promoting accumulation of provitamin A carotenoids in edible tissues. However, the mechanism by which Z-ISO mediated isomerization was unknown and it was unclear whether Z-ISO functioned independently as an isomerase. Here we present data to demonstrate that Z-ISO is a bona fide enzyme that catalyzes isomerization through a unique mechanism requiring a redox-regulated heme cofactor. This discovery raises new questions regarding control of carotenogenesis in plants.
2.3 Results

2.3.1 Expression, isolation, and activity assays of Z-ISO. We previously demonstrated that Z-ISO is essential for isomerization of a 15-cis carbon-carbon double bond in ζ-carotene by showing that when *E. coli* cells produced the Z-ISO substrate 9,15,9’ tri-cis-ζ-carotene, addition of a Z-ISO gene led to conversion of the substrate to 9,9’-di-cis-ζ-carotene (Chen et al., 2010). This isomerization activity occurred in the presence of several upstream carotenoid biosynthetic enzymes needed to produce the Z-ISO substrate. Thus, there remained the possibility that Z-ISO was not an enzyme itself but instead altered the activity of one of these other enzymes to create an enhanced function of isomerization. To directly test whether Z-ISO was a *bona fide* enzyme, we developed an *in vitro* assay using isolated Z-ISO from *Zea mays* and artificial liposomes containing the Z-ISO substrate. First, the substrate was purified from *E. coli* (Chen et al., 2010) and combined with lipids to form artificial liposomes. Next, we over-expressed and purified Z-ISO as a TEV protease-cleavable, maltose binding protein (MBP) fusion (MBP::Z-ISO) (Fig. 2A). Lastly, the isolated fusion protein of 90% purity (Fig. 2B) was incubated with TEV protease to cleave Z-ISO away from the fused MBP, prior to initiation of the isomerization reaction under reducing conditions. As shown in Fig. 2C, conversion of the substrate to product (seen as an increase in the ratio of 9,9’-di-cis-ζ-carotene/9,15,9’ tri-cis-ζ-carotene over background levels) occurred only in the presence of Z-ISO, as compared to reactions containing heat-denatured Z-ISO. Z-ISO catalyzed isomerization only when the reaction was conducted under reducing conditions but not oxidizing conditions. The liposomes used for the *in vitro* assay were absolutely essential to facilitate isomerization by the hydrophobic Z-ISO enzyme and for solubilization of the hydrophobic substrate and product.
2.3.2 Structural predictions. To gain insight into the mechanism of isomerization, we sought to identify catalytic motifs or other characteristic domains in Z-ISO. Our previous BLAST analysis (Tatusova and Madden, 1999) suggested that although Z-ISO is highly conserved in plants, it only shares sequence homology (~76% similarity) with NnrU, an uncharacterized membrane protein associated with nitric oxide metabolism in noncarotenogenic bacteria that perform denitrification (Chen et al., 2010). In addition, a chloroplast targeting sequence was identified in Z-ISO, suggesting that Z-ISO is a plastid-localized protein (Chen et al., 2010). No other motifs could be identified to suggest a mechanism for isomerization. Therefore, we began to apply a number of bioinformatic approaches to generate testable hypotheses on the location and function of Z-ISO.

We first utilized several topology-prediction programs and found that predictions of transmembrane (TM) domains in Z-ISO varied. MEMSAT3 (Jones, 2007), which has been experimentally validated as one of the better predictors of membrane topology (ter Horst and Lolkema, 2010) predicted seven TM domains in maize Z-ISO (Fig. 3) with TM 2-7 showing homology to the corresponding TM domains in NnrU. In comparison to a functional Arabidopsis transcript (ZISO1.1), a shorter Arabidopsis transcript (ZISO1.2) (Chen et al., 2010) encodes a nonfunctional protein with one less TM domain at the C-terminus. The effect of the deletion suggests that the C-terminal TM domain is important for the function (e.g. activity or proper folding) of Z-ISO.
To test the prediction that Z-ISO is targeted to the chloroplast, we fused the gene encoding green fluorescent protein (GFP) downstream of the gene encoding Z-ISO, including its transit peptide. The fusion construct was then transiently expressed in maize leaf protoplasts. GFP fluorescence confirmed that Z-ISO co-localized in the chloroplast together with chlorophyll (Fig. 4A). *In vitro* chloroplast protein import demonstrated that Z-ISO is a chloroplast integral membrane protein (Fig. 4B), as predicted by the topology predictions. Taken together, our observations suggest that Z-ISO is localized in chloroplast membranes. We also found that Z-ISO exists in a high molecular weight protein complex of about 480 kDa (Fig. 4C) as similarly noted for other carotenoid enzymes (Maudinas et al., 1977; Camara et al., 1982; Kreuz et al., 1982; Al-Babili et al., 1996; Bonk et al., 1997; Lopez et al., 2008).

Next, we applied homology modeling tools to look for structural homologies missed by the BLAST analysis. We expected that homology modeling would be limited by the underrepresentation of membrane protein structures in the Protein Data Bank due to inherent difficulties in crystallizing membrane proteins. Homology modeling of Z-ISO using the Meta Server (Ginalska K, 2003) program modeled the residues of Z-ISO onto an integral membrane protein, the diheme cytochrome b subunit of quinol:fumarate oxidoreductase (Lancaster et al., 2001; Madej et al., 2006). The fold recognition program “LOOOP”(Brinda Kizhakke et al., 2009) predicted that Z-ISO might contain nonheme iron. However, neither NnrU nor Z-ISO had been annotated as metalloproteins.
2.3.3 Detection of iron in Z-ISO. To test the prediction that Z-ISO is a metalloenzyme, inductively coupled plasma optical emission spectrometry (ICP-OES) was used to measure the metal content. The result showed that iron is present in the MBP::Z-ISO fusion, but not Ca, Cu, Mg, Mn, Mo, or Zn. Since MBP is not a metalloprotein, the protein-bound iron was postulated to be exclusively associated with Z-ISO. Cultures with MBP::Z-ISO are brown (Fig. 5A), as is the purified protein (Fig. 5B), consistent with the presence of heme or nonheme iron.

2.3.4 Detection and characterization of a heme b cofactor. To test for heme, MBP::Z-ISO and cleaved Z-ISO and MBP were separated by SDS-PAGE and stained for heme (Thomas et al., 1976) and then for total protein. The results showed that both MBP::Z-ISO and Z-ISO contained heme, while MBP did not (Fig. 5C). We next conducted a pyridine hemochrome assay (Berry and Trumpower, 1987) to examine the heme cofactor independent of Z-ISO, and found that it is a heme b on the basis of the spectroscopic signature (Nygaard et al., 2006) (Fig. 5D). We also found that the related NnrU protein contains a heme b (data not shown). Optical spectra (Fig. 6A) of “as-purified” Z-ISO, together with its bound heme, indicated the presence of an oxidized, ferric Fe(III) state, heme. To generate the spectrum of the reduced Z-ISO heme (with a ferrous, Fe(II), heme iron), the “as-purified” Z-ISO was treated with dithionite. While the spectrum of the “dithionite-reduced Z-ISO” (Fig. 6A and B) is similar to those of cytochromes containing heme b with two axial histidine ligands (Cheek et al., 1999; Uno et al., 2004; Owens et al., 2012), an “exogenous ligand”, carbon monoxide (CO), was shown to coordinate to the heme iron of Z-ISO (Fig. 6A and B). The binding was stoichiometric, given that the absorbance of the band trough at 560 nm in the CO difference spectrum (Fig. 6) was ~90% the intensity of the band.
peak for the same sample in the reduced minus oxidized spectrum. The comparison indicated that at least 90% of the reduced heme had bound CO. This result suggested that one of the axial amino acid ligands may be displaced by an exogenous ligand. The significance of this observation is that the Z-ISO heme iron may not be limited to shuttling of electrons as in the case of hemes that participate in electron transfer, but instead the Z-ISO heme iron may serve a role in catalysis.

2.3.5 Further evidence for the rearrangement and displacement of an axial ligand of the heme. Ultraviolet-Visible (UV-Vis) absorption spectroscopy analysis showed that an exogenous ligand can bind to the heme iron in the Fe(II) state but it was not known whether an exogenous ligand can displace an axial ligand when the heme is in the Fe(III) state. To test this possibility we introduced cyanide (CN$^-\$), which is known to bind preferably to ferric rather than ferrous heme (Boffi et al., 1997). CN$^-$ was added to both the “as-purified” enzyme with “oxidized” Fe(III) heme and to the dithionite-reduced enzyme carrying a ferrous heme, and the UV-Vis absorption was measured as shown in Fig. 7. Binding of CN$^-$ to heme was assessed through the spectral changes observed in a difference spectrum of “oxidized + CN$^-\$” minus "oxidized". Binding of CN$^-$ to the Fe(III) heme of Z-ISO using saturating concentrations of KCN was observed as indicated by the shift in the Soret peak (from 413 to 415/416 nm). The Fe(III)-CN complex of MBP::Z-ISO showed similar spectral shifts as for cyanomyoglobin which also has histidine as a proximal ligand (Tsai et al., 1993). Addition of CN$^-$ to the ferrous enzyme showed no spectral change (data not shown), as expected (Boffi et al., 1997). These results support the presence of a pentacoordinate, high spin mono-His liganded ferric heme which can bind
exogenous ligand in the oxidized, inactive enzyme. The sub-stoichiometric binding of CN\(^-\) suggests that this pentacoordinate, high-spin species represents a subset of the total ferric heme.

**2.3.6 Electron spin resonance (EPR) detects multiple heme species in Z-ISO.** An X-band EPR spectrum of the “as-purified” MBP::Z-ISO fusion protein indicated the presence of a high-spin ferric heme (i.e., heme \(b\) with an axial histidine ligand) at \(g = 5.8\) and multiple low-spin hemes with broad EPR signals (Fig. 8A and Table 1). In addition, a minor nonheme iron species was observed at \(g = 4.3\) and is postulated to be non-specifically bound to Z-ISO. As summarized in Table 1, the low-spin heme EPR signals shown in Fig. 8B are consistent with the existence of two major types of low-spin heme species with either a \(bis\)-His or His-Cys axial ligand set, respectively. The low-spin species at \(g = 2.98\) is assigned as a hexacoordinate heme with a \(bis\)-His axial ligand set, based on the similarity of its \(g\)-factors to those of other heme species with a \(bis\)-imidazole axial ligand set (Walker, 2004; Zoppellaro et al., 2009; Geng et al., 2012). The signals at \(g = 2.54, 2.50,\) and \(2.43\) are attributed to the \(g_x\) tensors for multiple components of a hexacoordinate low-spin heme species with a His-Cys axial ligand set (Smith et al., 2012; Zhong et al., 2014). Such low-spin species typically display a narrow distribution of the \(g\) factors, due to pronounced delocalization of the spin density to the cysteine ligand. The heterogeneity of this His-Cys coordinated heme species is likely originated from variations in the coordination position as well as the protonation or hydrogen-bonding state of the cysteine ligand. Previous studies on other systems have demonstrated that the \(g\) factors for His-Cys coordinated heme species are sensitive to the electronic properties of the heme environment and the protonation state of the axial ligands (Smith et al., 2012; Zhong et al., 2014). Next, we chemically reduced
the “as-purified” sample with dithionite and treated the reduced sample, which was EPR silent (Fig. 8C). With addition of nitric oxide (NO), a strong EPR signal at the $g = 2$ region was detected (Fig. 8D), and is attributed to the formation of a low-spin hexacoordinate Fe(II)-nitrosyl heme complex (Enemark and Feltham, 1974). NO binding is consistent with the finding that reduced MBP::Z-ISO also binds CO (Fig. 6). The EPR spectrum of the Fe(II)-nitrosyl complex of MBP::Z-ISO is similar to other Fe(II)-nitrosyl adducts of histidine-ligated hemes, such as those reported in cytochrome $c$ oxidase, cytochrome $c$ peroxidase, heme oxygenase, hemoglobin, myoglobin, and horse radish peroxidase (Yonetani et al., 1972; Sun et al., 1993; Collman et al., 2008), suggesting that a histidine residue is retained as the axial ligand of the ferrous heme when NO is bound.

2.3.7 Magnetic Circular Dichroism (MCD) confirms the low spin heme. EPR analysis revealed high-spin and low-spin hemes. A high spin heme can have an easily observable EPR signal, even if it is a minor component. To further examine the heme(s) in the same sample as used for EPR, we utilized Magnetic Circular Dichroism (MCD) which detects mainly the heme chromophore (300 - 700 nm). To ascertain a detection limit of the percentage of high spin heme in a sample containing a mixture of low spin and high spin heme, we compared the MCD and UV-Vis absorption spectra of Fe(III) Cyt. $b5$ (100% low spin species) (Vickery et al., 1976) and Fe(III) Mb (met-aqua-Mb) (~100% high spin) (Vickery et al., 1976) in a series of low spin/high spin mixtures (95/5, 90/10, 80/20 and 50/50) (Supplementary Fig.1). We concluded that Fe(III) Z-ISO (Fig. 8A) contains <20% (probably <10%) high spin heme at ambient temperature, presumably from equilibrium dissociation of an axial ligand from the low spin heme.
2.3.8 Cys and His as reversible heme ligands. MCD also showed that ferric Z-ISO has two ligand pairs (His/His and His/Cys), consistent with the EPR results. This finding was determined by comparing the Z-ISO spectrum with that of a simulated mixture of Cyt. b5 (bis-His) (Vickery et al., 1976) and imidazole (Im)-bound P450CAM (His-Cys) (Dawson et al., 1982; Sono et al., 1986) (Fig. 9, “oxidized”). The data show a good fit to two ligand coordination modes in low spin ferric Z-ISO at a ~1:1 ratio. If there is only one heme center in the protein, His and Cys might occupy the distal side of the heme as alternative ligands while the proximal side is ligated by a common His. If two heme centers exist, there should be two separate proximal histidines. To distinguish between these alternate hypotheses it was necessary to use another approach to identify all histidines in Z-ISO that could serve as heme ligands.

2.3.9 Identifying specific heme ligands in Z-ISO. We next searched for the specific residues that might function as Z-ISO heme ligands. We aligned available Z-ISO sequences, identified all evolutionarily conserved residues that have been reported to serve as heme ligands (Li et al., 2011), mutagenized them to alanine, and tested for activity using E. coli complementation (Chen et al., 2010). Of all conserved histidines, only two (H150, H266) were required for activity (Fig. 10). Substitution with alanine at H150 (“H150A”) or H266 (“H266A”) decreased the conversion of the substrate to product as compared to wild-type Z-ISO. Loss of the isomerization activity was not due to absence of expression, the possibility of which was ruled out using an anti-maize Z-ISO polyclonal antiserum (Supplementary Fig. 2). Loss of either residue also disrupted heme-binding as evidenced by the reduction in bound heme for MBP.
fusion proteins carrying the alanine variants and by the UV-Vis spectral shift seen for both the “as-purified” (oxidized) or dithionite-reduced proteins (Fig. 11; Table 2).

On the basis of the mutagenesis results, we were able to rule out the two-heme model for Z-ISO. Two hemes would have necessitated a total of at least three required histidines (two proximal and at least one distal), but we found no additional conserved histidines required for activity (Beltrán and Wurtzel, unpub.) beyond H150 and H266. Predicted locations of H150 and H266 based on the Z-ISO homology model (Fig. 12A) are consistent with coordination of a common cofactor. We also examined the MCD spectrum of the dithionite-reduced Z-ISO. MCD showed a single heme species in the reduced Z-ISO coordinated by bis-His (Fig. 9). Importantly, the amount of reduced His/His heme was equivalent to the combined concentration of the His/His and His/Cys heme seen in oxidized Z-ISO. Therefore the data are consistent with the presence of a single heme that undergoes a change in axial ligation when reduced (Fig. 12B).

2.3.10 Does the heme ligand C263 function in isomerization? The ability of Z-ISO to bind exogenous ligands (as summarized in Fig. 12B and demonstrated above) indicates availability of an axial coordination site on its heme, and facile dissociation of one of two axial histidine ligands. The most complete dissociation takes place in the reduced, active form of the enzyme. Z-ISO activity is predicated on the heme ligands H150 and H266 and on the heme iron being in the reduced state. Therefore, it is possible that the heme iron directly mediates isomerization by interacting with the substrate. An alternative hypothesis is that as a result of redox-dependent ligand switching, the switch to bis-His exposes C263 which becomes accessible to mediate
catalysis. Precedence for the function of a Cys residue in catalysis, particularly in double bond isomerization, is seen for isopentenyl diphosphate (IPP) isomerase (IPPI), a nonheme enzyme that catalyzes double bond isomerization (Durbecq et al., 2001; Wouters et al., 2003). The C263 alternate heme ligand is the only cysteine in Z-ISO and it is evolutionarily conserved in all Z-ISO sequences. We do not believe that the Cys functions in protein dimerization as our *in vitro* reaction included the reducing agent, dithiothreitol, which did not inhibit the reaction. If C263 is essential for catalysis, then mutagenesis to a non-redox active residue should inactivate the enzyme. As shown respectively in Fig. 10 and Supplementary Fig. 2, mutation to alanine had no effect on activity or expression, when using the *E. coli* complementation system. While the C263A MBP fusion variant carries a reduced amount of heme equivalent to the H266A variant, the UV-Vis spectrum of C263A is similar to wild type Z-ISO (Fig. 11; Table 2). Taken together, these results suggest that C263 is not catalytic but instead plays a role in heme binding and reversible heme ligation.

2.4. Discussion

2.4.1 Z-ISO is a *bona fide* enzyme

Our data provide definitive proof that Z-ISO is a *bona fide* enzyme that mediates the *cis* to *trans* double bond isomerization of 9,15,9′-*cis*-ζ-carotene (the product of PDS) to form 9,9′-*cis*-ζ-carotene (the substrate for ZDS). Z-ISO contains a single heme *b*. The heme is likely to function as the mechanistic cofactor based upon the observations that Z-ISO is inactive with loss of either of the apparent His ligands (H150 or H266). The heme *b* in “as-purified” Z-ISO is ferric and primarily low-spin. MCD spectroscopy identifies the axial ligands as *bis*-His or His-Cys in a 1:1
ratio. It should be noted that H266 and C263 are only three residues apart. Therefore, these two residues are likely the labile ligands that can exchange with each other in the ferric state, whereas H150 is the tightly associated ligand that always remains bound to the heme regardless of different redox or binding events (Fig. 12). EPR spectra show a small amount of His-ligated, pentacoordinate, high-spin heme, a possible intermediate during the ligand exchange. The presence of this high-spin species with a coordination vacancy, in equilibrium with the two different hexacoordinate ligation states of the low-spin heme, is consistent with the observation that CN⁻ can bind to the ferric heme of Z-ISO. Furthermore, the sub-stoichiometric binding of CN⁻ is consistent with the MCD calibration data (Supplementary Fig. 1) showing that the pentacoordinate, high-spin species is likely to be less than 10-20% of the total heme. When Z-ISO is reduced to the Fe(II) form, the heme ligand set becomes solely bis-His, suggesting a redox-dependent ligand switch (Aono et al., 1998; Allen et al., 2000; Igarashi et al., 2008; Marvin et al., 2008; Smith et al., 2012). It is this reduced form that is active in vitro. The Fe(II) heme can bind NO and CO, revealing the ability of the reduced heme to coordinate an exogenous ligand (Fig.12B).

2.4.2 Proposed mechanism for Z-ISO catalysis of isomerization

We propose that the redox-dependent ligand switch can cause conformational changes at the active site of Z-ISO. The conformational changes may function as a redox-regulated trigger to control the binding of the bulky substrate (Fig. 1). In the resting ferric state, Z-ISO is postulated to be in a “closed” conformation, excluding the binding of its substrate. Reduction of the heme iron switches coordination of the heme to bis-His and exposes the active site for substrate
binding. In the proposed mechanistic model (Fig. 13), binding of the Z-ISO substrate displaces the weakly associated H266 ligand, and the π electrons of the 15-15\textsuperscript{\prime} \textit{cis} carbon-carbon double bond in the substrate serve as a Lewis base for coordination with the ferrous heme iron of Z-ISO. There is precedence for coordination between a carbon-carbon double-bond moiety and a heme iron as reported for a bacterial flavohemoglobin (D'Angelo et al., 2004). In that study, spectroscopic evidence provided support for coordinate bonding between the iron of the histidine-coordinated heme and a carbon-carbon double bond of an unsaturated lipid. As a result of direct coordination between the ferrous heme iron of Z-ISO and the target double-bond in the substrate, the single sigma bond remaining in the substrate would be free to rotate to the energetically more favorable \textit{trans} configuration, thus converting 9,15\textsuperscript{\prime},9\textsuperscript{\prime}-\textit{cis}-ζ-carotene to 9,9\textsuperscript{\prime}-\textit{cis}-ζ-carotene. As a consequence of \textit{cis} to \textit{trans} isomerization, the entire structure of the 40-carbon ζ-carotene substrate would change from a bulky W-shape to a streamlined linear shape. These \textit{cis} and \textit{trans} geometrical isomers would interact uniquely with the microenvironment of the Z-ISO protein structure and contribute distinctly to membrane lipid fluidity. Therefore, it is predicted that the altered carotenoid structure would drive release of the product from Z-ISO, allowing further enzymatic conversions of the Z-ISO product by downstream enzymes. Notably, according to the hard-soft acid-base (HSAB) theory (Pearson, 1963, 1968), Fe(II) is a soft Lewis acid compared to Fe(III), and thereby prefers ligation to soft Lewis bases. Given that the Z-ISO substrate is a soft Lewis base, it is anticipated that the ferrous state of Z-ISO presents superior binding kinetics and reactivity compared to the ferric state. Thus, other than the aforementioned redox-dependent conformational changes, this HSAB analysis provides an additional layer of molecular basis for the redox control of the isomerization activity of Z-ISO.
Heme-dependent carbon-carbon double-bond isomerization is rarely reported in the literature. The only other double-bond isomerase known to utilize heme as a cofactor is a bacterial *cis-trans* fatty acid isomerase (CTI) (Holtwick et al., 1999; Heipieper et al., 2003; Heipieper et al., 2004). CTI is a periplasmic enzyme that utilizes a *c*-type heme to perform a similar *cis* to *trans* isomerization of a double bond. However, little is known regarding the electronic structure or ligand coordination state of the heme iron in this enzyme. The hypothesized catalytic mechanism of CTI is distinct from that of Z-ISO. It is proposed that CTI functions in the oxidized ferric state and that the isomerization reaction is triggered by single-electron transfer from the double bond to the heme iron, oxidizing the double bond to single bond (Heipieper et al., 2004).

### 2.4.3 The role of the ligand switch in Z-ISO and other hemoproteins.

Our data show that reduction of the Z-ISO heme iron from Fe(III) to Fe(II) is necessary for enzyme activity. The heme reduction causes a ligand switch to *bis*-His and possibly triggers additional conformational changes at the active site of Z-ISO to allow substrate binding. Such redox-dependent ligand-switch phenomena have been observed in many other hemoproteins, and the purpose of the ligand-switch behavior is to induce conformational changes that drive functional activation, similar to the scenario in Z-ISO (Aono et al., 1998; Allen et al., 2000; Marvin et al., 2008; Smith et al., 2012). This strategy appears to be a common natural approach to control the functional activity of hemoproteins through redox changes. For example, cytochrome *cd*1 nitrite reductase must be reduced to become catalytically active through a mechanism that involves a redox-mediated heme iron ligand switch (Williams et al., 1997). Upon reduction, a tyrosine ligand of the *d*1 heme in that enzyme is displaced to generate a coordinate vacancy for substrate binding.
Similarly, the CO gas sensing transcription factor CooA contains a heme cofactor that undergoes a ligand switch in order for CooA to become competent for DNA binding (Smith et al., 2012). Like Z-ISO, CooA goes through a redox-mediated ligand switch upon reduction of the heme iron; a cysteine axial ligand is replaced by a histidine, enabling the binding of CO to the heme iron at the ferrous state via displacement of the relatively weakly bound histidine ligand. Conformational changes then follow to drive DNA binding. Another example is bacterial di-heme cytochrome c peroxidase (bCcP) (Geng et al., 2014). In the resting di-ferric state of bCcPs, one heme has a bis-His axial ligand set and the other heme has a His-Met axial ligand set. The two hemes are over 14 Å apart. A “reductive activation” process is generally needed for the proper function of bCcPs: single-electron reduction of the high-potential His-Met heme triggers a series of conformational changes that remotely displaces one of the histidine ligands of the other heme, allowing the access of the co-substrate, H$_2$O$_2$, to that site. Notably, a common feature of these examples is that reduction of the inactive ferric form generates the active ferrous form and the ligand switch as well as associated conformational changes enables the binding of substrate via the creation of a coordinate vacancy/weekly associated ligand and/or a binding cavity. This strategy can effectively protect the heme cofactor from non-productive binding events and thereby avoids undesired side reactions.

### 2.4.4 Controlling Z-ISO activity through plastid redox status

If the activity of Z-ISO is controlled by redox state, then how might plastid physiology and stress affect Z-ISO and downstream flux through the carotenoid pathway? Plastids undergo dramatic shifts in redox status as a result of photosynthetic activity in the light and nonphotosynthetic...
activity in the dark. It is known that changes in redox status are reflected through dynamic control of metabolism. For example, redox modulators (e.g., ferredoxins and thioredoxins) adjust heme and chlorophyll biosynthetic activity in response to varying redox state (Richter and Grimm, 2013). It has been proposed that carotenoid biosynthesis is also under redox control, although most of the molecular details are unknown (Josse et al., 2000; Nashilevitz et al., 2010; Fanciullino et al., 2014). Based on the results presented here, we predict that changes in plastid redox state will directly influence Z-ISO activity, and as a consequence alter flux in the carotenoid biosynthetic pathway.

2.4.5 Nitric oxide and Z-ISO

NO is known to inhibit heme enzymes through binding to the heme iron (Khatsenko et al., 1993; Khatsenko, 1998), especially the ferrous form (Cooper, 1999). It is known that NO is produced directly at the site of carotenoid biosynthesis in plant plastids in response to stress (Gas et al., 2009; Zhao et al., 2009) and has been shown to inhibit carotenoid accumulation (Chang et al., 2013). The ability of Z-ISO to bind NO suggests that Z-ISO is potentially regulated by NO in vivo. Stress is a known factor affecting biosynthesis and action of carotenoids and their derivatives (Davison, 2002; Johnson et al., 2007; Li et al., 2008a; Li et al., 2008b; Walter et al., 2010; Zhang et al., 2010). Carotenogenesis may be controlled by NO-dependent modulation of Z-ISO activity under certain stress conditions. Alternatively, NO might function as a gas sensor or provide another regulatory function by modulating the heme-ligand coordination or iron chemistry (Ascenzi et al., 2010). Further study of Z-ISO will be critical for advancing our limited understanding of post-translational regulation of carotenogenesis.
2.4.7 What is the significance of a heme isomerase?

Hemoproteins possess a wide range of biological functions, as enzymes, electron transporters, gas sensors, gas transporters, and as transcription factors, but double-bond isomerization is not generally considered a prototype activity for hemoproteins (Munro et al., 2009). Z-ISO is the only known heme-dependent isomerase that utilizes a ferrous iron, undergoes redox-mediated ligand switching, and performs isomerization in a membrane environment. Therefore, studies of Z-ISO as presented here open the path for further discovery and understanding of a new class of hemoenzymes that perform double-bond isomerization in hydrophobic environments. In the case of Z-ISO, isomerization is critical for mediating metabolic flux of a vital plant pathway that is also of importance for human and animal nutrition. Z-ISO is unique in that isomerization of a long hydrocarbon is performed in a hydrophobic environment and substrate and product have dramatically different properties affecting membrane fluidity. These properties of Z-ISO are valuable for bioengineering such that in the future it will be possible to evolve new types of Z-ISO enzymes to create new chemistries and altered membrane compositions. Further understanding of Z-ISO function will provide opportunities to better control carotenoid biosynthesis for breeding more resilient plants in a changing climate and to facilitate production of more nutritious crops.

2.5 Methods

For detailed protocols related to the methods described, see: Appendix 1 (Z-ISO expression and purification; Appendix 2 (Z-ISO in vitro enzyme assay); Appendix 3 (Separation of ζ-carotene
isomers by HPLC); Appendix 4 (Generation of antibodies against Z-ISO); Appendix 6 (Plasmids used in this study and laboratory clone sheets).

2.5.1 Z-ISO localization

2.5.1.1 Transient expression of Z-ISO in protoplasts. Transient expression of Z-ISO in protoplasts. A full copy of maize Z-ISO without a stop codon was amplified from pColZmZ-ISO1 plasmid (#497) (Chen et al., 2010), with forward primer 2793 (5’ atctctagaATGGCCTCCCAGCTCCGCCTCCACC), containing an XbaI site, and reverse primer 2794 (5’ atcggatccCCAGGGAAGTTGGTAGCTGGATGC), containing a BamHI site, and inserted into the pUC35S-sGFP-Nos vector (Shumskaya et al., 2012) (digested with XbaI/BamHI), to produce the pUC35S-M-ZISO-sGFP-Nos plasmid (#568) which was used for transient expression. Transient expression of Z-ISO-GFP in maize green leaf protoplasts was performed as described (Shumskaya et al., 2012).

2.5.1.2 In vitro import of Z-ISO into chloroplasts. In vitro import of Z-ISO into chloroplasts. A full copy of the maize Z-ISO gene, without a stop codon, was amplified from pColZmZ-ISO1 (#497) (Chen et al., 2010) using forward primer 2851 (ccacctgcaGAATTCtatggcctc), containing an EcoRI site, and reverse primer 2854 (gtcTCTAGAattttttcaatgtgaggtgagaccaccagggaagtggtagct), containing a Strep-tag and XbaI site, and inserted into vector pTnT (Promega), which was digested with the same restriction enzymes, to yield plasmid pTnT-M-ZISO-Strep (#570). pTnT-M-ZISO-Strep was used as a
template for *in vitro* protein synthesis. *In vitro* protein synthesis and import of Z-ISO into isolated pea chloroplasts were performed as described (Quinlan et al., 2012).

### 2.5.1.3 Identification of a Z-ISO complex

After $^{35}$S-met-labelled Z-ISO was imported into chloroplasts, the chloroplast sample was treated with 0.5% Triton X-100 to isolate protein complexes under native conditions. The sample was then separated into individual complexes by native gel electrophoresis in a NativePAGE Novex 4-16% gel (Invitrogen, Life Technologies), following the instructions of the manufacturer. The gel was then dried and the radioactive band detected by a Phosphorimager system (Amersham, GE Life Sciences). The size of the band was estimated in comparison to NativeMark protein marker (Invitrogen, Life Technologies).

### 2.5.2 Z-ISO expression and purification

#### 2.5.2.1 Cloning

The maize Z-ISO coding sequence with transit sequence was commercially synthesized (Genscript, Piscataway, NJ) to be codon optimized for *E. coli* and restriction sites added for cloning into *Sae*I and *Bam*H1 sites of pUC57 (see sequences in Supplementary Data). The final construct was named ZmZISO ACA-less (# 516). From this clone, the sequence encoding Z-ISO beginning at residue 49 was PCR amplified using primers (forward) tacttcaatccatgcctcg TGCCGGCGCGGGGTGG and (reverse) TTATCCACTTCTGCTG and inserted by ligation independent cloning (LIC) (Doyle, 2005) into pMCSG9-10xHis (# 646). Primer sequences in lower case letters were for LIC cloning and those in uppercase were gene-specific. The resulting construct, pMCSG9 Z-ISO E2 (# 582), encodes a MBP::Z-ISO fusion protein consisting of a 10x-His-tagged maltose
binding protein (MBP) at the N-terminus which is separated from the C-terminal Z-ISO by a TEV protease cleavage site. The pMCSG9-10xHis vector was produced by modifying vector pMCSG9 (Donnelly et al., 2006) and obtained from the materials repository of the Protein Structure Initiative (Seiler et al., 2014) to have a 10x His tag instead of a 6xHis tag.

2.5.2.2 Expression and purification of the MBP::Z-ISO E2 fusion protein. E. coli C43 (DE3) overnight cultures harboring pMCSG9 Z-ISO E2 (# 582) were used to inoculate 2 X YT medium (1% yeast extract, 1.6% tryptone and 0.5% NaCl) at 1:100 dilution. Cultures were incubated with shaking at 200 rpm at 37°C until an O.D. of 0.6 (typically ~2 h). Protein expression was induced with 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and further incubated for 16 h at 28°C. Cultures were centrifuged at 2,600 x g for 15 minutes at 4°C and pellets frozen until use. Pellets were resuspended (at a ratio of 50 mL per 8 g of cell pellets, (~ 40 mL per liter of initial culture) in Resuspension Buffer (50 mM Tris pH 7.6, 300 mM NaCl, and 5% glycerol) containing 0.5 mM dithiothreitol (DTT), 4 μL/25 mL benzonase, (Sigma-Aldrich) and 60 mg/50 mL of 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 0.15 mg/mL of lysozyme (Sigma-Aldrich) before sonication on ice (5 times, 30 sec each, 60% power) using a Vibra Cell VC600 sonicator equipped with a 3 mm tapered microtip (Sonics & Materials Inc, Connecticut, USA) To remove unbroken cells, the preparations were centrifuged at ~15,000 x g (11,000 rpm in a Type 45 Ti rotor) for 15 minutes at 4 °C. To recover the membrane fraction, the supernatants were next centrifuged at ~120,000 x g (32,000 rpm in a Type 45 Ti rotor) for 1 h at 4 °C. The pellets containing cell membranes were resuspended in Resuspension Buffer at a ratio of 8 mL per liter of initial cell culture, following by sonication as described above. Following sonication,
the volumes were increased for a total of 40 mL per liter of starting culture. n-Dodecyl β-D-maltoside (DDM [Anatrace]), added as powder was added to a final concentration of 1.5%. Samples were rotated end over end at 4°C for 15 min. Cleared lysates were incubated overnight with Ni-NTA containing resin (Qiagen) at a ratio of 300 µL resin per 40 mL of lysate for Immobilized Metal Affinity Chromatography (IMAC) in a 5 mL polypropylene column (Qiagen). The column was washed with five resin volumes of ATP Wash Buffer (40 mM Tris pH 7.6, 200 mM NaCl, 5% glycerol, and 5 mM MgCl₂), containing freshly added final concentrations of 5 mM ATP, 0.1 mM DTT and 0.05% DDM for 30 min (column under gentle rotation). A second wash (5 resin volumes) with Wash Buffer (40 mM Tris pH 7.6, 400 mM NaCl, 5% glycerol) containing 0.1 mM DTT, 0.05% DDM and 30 mM histidine was performed for 5 min (column under gentle rotation). The MBP::Z-ISO fusion protein was eluted with Elution Buffer (25 mM Tris pH 7.6, 200 mM NaCl, 200 mM histidine and 5% glycerol) containing 0.1 mM DTT and 0.05% DDM at a ratio of 1 mL Elution Buffer per liter of initial culture. The protein sample was then dialyzed overnight using a Slide-A-Lyzer Dialysis cassette G2 20 K membrane,(Thermo-scientific, IL, USA) against 1000-fold volume of buffer containing 20 mM NaCl, 20 mM Tris (pH 7.6), 5% glycerol, 0.02% DDM, and 0.1 mM DTT at 4°C. For metal analysis, 1 mM EDTA was included in the dialysis buffer and dialysis was done for 3 h, three times. When needed, the sample was concentrated using micro-concentrators (Microcon micro-concentrators 100 K, Amicon, Inc., Beverly, MA USA). For in vitro assays, protein was stored at -20°C in buffer containing 20 mM NaCl, 20 mM Tris (pH 7.6), 40% glycerol, 0.02% DDM, and 0.1 mM DTT. The yield of fusion protein was ~1 mg/liter culture at ~90% purity (see Appendix 1 for a detailed protocol).
2.5.3 Z-ISO *in vitro* enzyme assay

2.5.3.1 Preparation of substrate-containing liposomes. To produce the substrate, 9,15,9'-tri-cis-ζ-carotene ("tri") from *E. coli* BL21 (DE3) cultures, 400 mL of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing chloramphenicol (34 μg/mL) was inoculated with 8 mL of overnight culture containing pACCRT-EBP (#150). Cultures were grown in the dark at 37°C, with shaking at 160 rpm for 8 h before induction with 10 mM IPTG. Cultures were further incubated at 28°C, with shaking at 100 rpm for 40 h and an additional 2 d without shaking. Cells were centrifuged at 2,600 x g and pellets were resuspended in a total of 40 mL of methanol, distributed in 4 Falcon tubes with equal volumes of extract and sonicated twice on ice, for 30 sec each at 60% power using a Vibra Cell VC600 sonicator equipped with a tapered 3 mm microtip (Sonics & Materials Inc, Connecticut, USA). Extracts were centrifuged at 2,600 x g for 10 min and supernatants were transferred to 15 mL Falcon tubes and evaporated under nitrogen gas in the dark. Dried samples were resolubilized in 300 μL of methanol, transferred to 1.5 mL microfuge tubes, frozen at 80°C for 1 h and centrifuged at 16,000 x g at 4°C. Extractions were then combined and 1 mL used to prepare liposomes. Cells also accumulate 9,9'-tri-cis-ζ-carotene ("di") and therefore enzymatic conversion is measured as the ratio of "di" to "tri" isomers. To prepare liposomes, one mL of substrate extract (58 μM, estimated by spectroscopy using the molar extinction coefficient for ζ-carotene (Britton et al., 1995); $\varepsilon_{400} = 138,000$) was mixed with 35 μL of soybean L-α-Phosphatidylcholine (Sigma-Aldrich) (20 mg/mL in methanol). The mixture was dried under N₂ followed by addition of 800 μL sonication buffer (25 mM HEPES pH 7.8, 100 mM NaCl, 10% glycerol) and sonicated on ice using a Vibra Cell VC600 sonicator equipped with a 3 mm tapered microtip (Sonics & Materials Inc,
Connecticut, USA) for 1 min at intervals of 10 s at 20% power (see Appendix 2 for a detailed protocol).

2.5.3.2 In vitro reactions. To assemble a biphasic assay system (final volume of 400 μL), purified, MBP::Z-ISO fusion protein (10 μM final concentration) was incubated with 15 μL of AcTEV protease (150 units, Invitrogen) for 2 min at RT. To generate reducing conditions, freshly prepared sodium dithionite was added to a final concentration of 10 mM in the assay. To initiate the reaction, 200 μL of substrate-containing liposomes, (for a final concentration of 36.5 μM substrate) were added and reactions were overlaid with N₂ gas before capping. Reactions were incubated at 28°C under continuous shaking at 130 rpm for 3 h in the dark (to prevent photoisomerization). Reactions in the absence of sodium dithionite were also assembled. As a negative control, heat denatured (10 min at 100°C) MBP::Z-ISO fusion was used. Reactions were extracted by addition of 1 mL of petroleum ether/diethyl ether 2:1 (v/v) and the organic phase collected, dried under N₂, dissolved in 150 μL methanol and 100 μL separated by HPLC as described below. All reactions were replicated three times (see Appendix 2 for a detailed protocol).

2.5.4 HPLC Analysis

HPLC separations were performed on a Waters HPLC system equipped with a 2695 separation module, 996 photodiode array detector (Waters), and Empower I software (Waters). A C30 Develosil 5u RPAQUEOUS (250 x 4.6 mm) column from Phenomenex (Nomura Chemical Co. Ltd, Seto, Japan) was used. For isocratic separation of 100 μL of carotenoid extract, a mobile
phase of four parts water, 66 parts methanol, and 30 parts methyl-t-butyl-ether at a constant flow rate of 1 mL/min for 80 min was applied. Identification of ζ-carotene isomers was based on elution time and spectra as published (Matthews et al., 2003; Li et al., 2007; Chen et al., 2010) (see Appendix 3 for a detailed protocol)

2.5.5 Detection of metals in Z-ISO

2.5.5.1 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Samples of Z-ISO were injected into a Spectro Genesis inductively-coupled optical emission spectrometer (ICP-OES) to measure the concentrations of iron at 238.204 nm and 259.941 nm and sulfur at 180.731 nm, as previously described (Varanasi and Hosler, 2012). No other metals were detected.

2.5.6 Detection of heme

2.5.6.1 Pyridine hemochrome assay. To determine whether the chromophore bound to Z-ISO was heme, a pyridine hemochrome assay was performed. Purified protein (750 µL) was mixed with 75 µL of 1 N NaOH, 175 µL of pyridine and 2 mg of sodium dithionite (Nygaard et al., 2006). The UV-visible absorption spectrum was immediately recorded and compared with the spectrum of the initial purified sample before addition of dithionite. The presence of the Soret band at 414 nm in the ferric state and the presence of the Soret band (418 nm) and appearance of the α/β bands at 555 and 530 nm respectively in the ferrous state were used as evidence for the presence of heme.
2.5.6.2 **Heme stain.** Heme staining, based on heme peroxidase activity, was performed essentially as reported (Thomas et al., 1976). Protein samples were separated on a NuPAGE® Bis-Tris 12% polyacrylamide gel (Invitrogen). The gel was rinsed with water for 15 s and then incubated for 1 h in the dark in a solution containing 30 mL of 40 mM TMBZ (3,3,5,5’-Tetramethylbenzidine, Sigma-Aldrich) in methanol followed by the addition of 70 mL of 0.25 M sodium acetate pH 5.0. Then, 5 mL of 3% hydrogen peroxide were added and mixed well until a signal corresponding to the MBP Z-ISO band appeared. The gel background was removed by destaining 15 min with 3:7 isopropanol: 0.25 M sodium acetate.

2.5.7 **Binding of CN**

A sample of 1 mL of MBP::Z-ISO (21 µM), purified as described above, was incubated with sodium cyanide (KCN, Sigma-Aldrich) at a final concentration of 2 mM. The UV visible spectrum was recorded before and immediately after addition and mixing of KCN. The experiment was repeated except that MBP::Z-ISO was first reduced with sodium dithionite (2 mg, added as dry powder) before addition of KCN.

2.5.8 **Electron spin resonance (EPR) spectroscopy**

X-band EPR spectra of Z-ISO were recorded in the perpendicular mode on a Bruker ER200D spectrometer coupled with a 4116DM resonator at 100 kHz modulation frequency. The measurement temperature was maintained at 10 K using an ESR910 liquid helium cryostat and an ITC503 temperature controller from Oxford Instrument (Concord, MA). The reduced Z-ISO protein was generated by dithionate reduction under anaerobic conditions. Nitric oxide (NO) was
anaerobically introduced through a gas-tight syringe to the headspace of the quartz EPR tubes containing reduced Z-ISO. An argon flush was maintained above samples to protect them from oxidation by O₂ and to minimize an anomalous EPR signal near g = 2 which derives from NO.

2.5.9 Magnetic Circular Dichroism (MCD)

MCD spectra were measured on a Jasco J815 spectropolarimeter fitted with a Jasco MCD-1B magnet at a magnetic field strength of 1.41 T at 4°C using a 0.5 cm pathlength quartz cuvette and interfaced with a Silicon Solutions PC through a JASCO IF-815-2 interface unit. MCD data acquisitions and manipulations were carried out using JASCO software as reported previously (Pond et al., 2000).

2.5.10 Site-directed mutagenesis and functional complementation

The maize Z-ISO cDNA coding sequence from pColZmZ-ISO1 plasmid (#497) (Chen et al., 2010) was used as a template to PCR amplify and subclone Z-ISO lacking the transit peptide sequence (amino acids 1-46). For PCR, forward primer (5’cgggatccctCACGCTCGTCCCCGCCCCGTGC 3’) containing a BamHI site and reverse primer (5’gcgtgaccTACCAGGGAAGTTGGTAGCT3’) containing a SalI site were used. Lowercase letters in primers contain restrictions sites and uppercase letters contain gene specific sequences. The resulting PCR product was further inserted into the BamHI and SalI sites of pCOLADuet-1 forming a His-tag::Z-ISO fusion and named pCola Zm Z-ISO NTP (#579). pCola Z-ISO NTP was then used as template to perform substitutions of conserved residues to Ala. Residue substitutions used in this study were: His-150 (#797, pCol Zm Z-ISO NTP H150A), His-266
(798, pCol Zm Z-ISO NTP H266A) and Cys-263 (796, pCol Zm Z-ISO NTP C263A). The same residues were mutated using the pMCSG9 Z-ISO E2 plasmid (582) as template to generate the following MBP::Z-ISO mutant versions: pMCSG9 Z-ISO E2 H150A (619), pMCSG9 Z-ISO E2 H266A (620) and pMCSG9 Z-ISO E2 C263A (801) which were expressed in E. coli as described above (see: “Expression and purification of the MBP::Z-ISO fusion protein”). Reactions for mutagenesis were performed using the Quick-change® Lightning Site-Directed Mutagenesis Kit (Stratagene) and primers designed to incorporate the desired substitution. For functional testing, the Z-ISO mutant genes were further transformed into E. coli cells harboring the plasmid pACCRT-EBP (150) which confers accumulation of ζ-carotene (Matthews et al., 2003). For functional complementation, 1 mL of saturated cultures in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) were added to 50 mL of fresh medium and then grown in the dark at 37°C at 200 rpm for 8h before induction with 10 mM IPTG and further incubation for 40 h at 28°C with slow shaking (100 r.p.m) and an additional 2 days without shaking. For carotenoid extraction, bacterial cultures were centrifuged at 2,600 x g for 10 min. Pellets were resuspended in 5 mL of methanol containing 1% of butylated hydroxytoluene (BHT) and sonicated using a Vibra Cell VC600 sonicator equipped with a 3 mm tapered microtip (Sonics & Materials Inc, Connecticut, USA) on ice twice, 30 sec each, 60% power. Extracts were centrifuged at 2,600 x g for 10 min and supernatants were transferred to 15 mL Falcon tubes and extracts evaporated under nitrogen gas in the dark. Dried samples were resolubilized in 500 µL of methanol, transferred to 1.5 mL microfuge tubes, frozen at 80°C for 1 h, centrifuged at 16,000 x g at 4°C, and supernatants used for HPLC separation as described above. Complementation experiments were replicated three times.
2.5.11 Immunodetection of Z-ISO

For antibody generation, 2 mg of MBP Z-ISO E2 protein (#582) were digested with TEV protease to generate free Z-ISO. Samples were separated using the NuPAGE system from Invitrogen, Carlsbad, USA). Protein bands corresponding to Z-ISO were sliced out and shipped to Lampire Biological laboratories for rabbit immunization. Polyclonal antibodies against Z-ISO were generated in 2 rabbits identified as 190202 and 190203. For immunodetection protein samples were separated by electrophoresis using the NuPAGE system (Invitrogen). Reducing conditions in the samples were generated with DTT (100 mM). Proteins were transferred onto nitrocellulose membranes (Optitran; Whatman, Dassel, Germany) using an electrophoretic transfer cell (Criterion Blotter, Bio-Rad) at 20 V overnight, 4 °C using 1 X transfer buffer (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol). The membranes were then incubated in blocking buffer [ 1X Phosphate Buffered Saline buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4 )], 3 % Bovine Serum Albumin (BSA) and 1 % Tween 20 for 1 h at RT, then with the rabbit (190203) anti-Z-ISO polyclonal antibody (1:2000) for 1 h at RT. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h at RT and washed with 1 X PBS buffer containing 1 % Tween 20 for 15 min followed by 4 additional washes of 5 min each. Immunoreactions were visualized with the Super Signal West Dura kit (Thermo Scientific). Fluorescent signals were captured using a G-box (Chemi XT4) from Syngene with Genesys V1.3.1.0 Software.
2.6. Acknowledgments

We thank Dr. Wayne Hendrickson (Columbia University and Principal Investigator, NIH-PSI New York Consortium on Membrane Protein Structure), for helpful discussions and use of the New York Structural Biology Center facilities. We thank Dr. Masayori Inouye (UMDNJ) for valuable advice on codon optimization and Dr. Louis Bradbury (Lehman College and CUNY) for helpful discussions. The Wurtzel lab was supported through funding from the National Institutes of Health (grant GM081160), City University of New York and Lehman College. The New York Consortium on Membrane protein structure was supported through funds obtained through from National Institutes of General Medical Sciences Protein Structure Initiative (PSI) program (grant GM095315). Funding of the Dawson lab was obtained from National Institutes of Health grant GM 26730; funding of the Liu lab was obtained from National Institutes of Health grant R01GM108988 and the Georgia Research Alliance Distinguished Scholar Program. Dr. Charles Ampomah-Dwamena prepared mutant Z-ISO fusion proteins while on a short-term sabbatical in the Wurtzel lab through funding by The New Zealand Institute for Plant and Food Research Limited, New Zealand.
Fig. 2.1. Z-ISO isomerization. Z-ISO catalyzes isomerization of the central 15-15’ C=C double bond of 9,15,9’-tri-cis-ζ-carotene, the product of PDS, to form 9,9’-di-cis-ζ-carotene, the substrate of ZDS.
Fig 2.2. Z-ISO *in vitro* enzyme assay using substrate-containing liposomes. For complete details, see Methods. A. Cartoon showing fusion protein containing Z-ISO and maltose-binding protein (MBP). B. Z-ISO fusion protein of 90% purity was analyzed by SDS-PAGE. MBP::Z-ISO (marked by arrow) was used for the *in vitro* assay (after cleavage) and for all subsequent spectroscopic analyses. Molecular weight markers (kD) are shown in the left lane. C. For the *in vitro* assay, purified MBP::Z-ISO fusion protein (FIG 2B) was incubated with TEV protease. To initiate the reaction, substrate-containing liposomes (with a starting ratio of ~0.8 di-cis/tri-cis isomers as indicated by the red dashed line) were added and incubated in the dark. To generate reducing conditions for the “reduced” active enzyme and “heat denatured” inactive enzyme, freshly prepared sodium dithionite was added in the assay. Oxidizing conditions were conducted in the absence of sodium dithionite. The *in vitro* reaction substrate and products were separated by HPLC and the ratio of product (“di-cis” is 9,9’ di-cis-z-carotene) and substrate (“tri-cis” is 9,15,9’ tri-cis-z-carotene) were measured using enzymes subjected to different treatments as noted. In the presence of liposomes containing excess substrate, the “reduced” Z-ISO shows a statistically significant conversion of substrate (“tri-cis”) to product (“di-cis”) as compared to oxidized or heat-denatured Z-ISO (3 replicates) which showed no increase over background levels.
Fig. 2.3. Maize Z-ISO topology predicted by MEMSAT3. The N-terminal transit sequence (1-46) predicted by ChloroP is not shown. TM, transmembrane domain; letters and numbers denote loops and residues, respectively. Residues discussed in this paper are highlighted. H150 and H266 are required for activity, whereas C263 is not required for activity.
**Fig 2.4. Z-ISO plastid localization.** A. Transient expression of Z-ISO in maize leaf chloroplasts. Z-ISO is co-localized with chlorophyll as seen in rightmost panel of “merged signals” (green GFP (Z-ISO) and red (chlorophyll autofluorescence) observed in a single cell). Bar=10 µm. B. *In vitro* import into pea chloroplasts. *In vitro* translated 38 kD Z-ISO is cleaved upon import to 33 kD, suggesting a 5 kD transit peptide. After import, chloroplasts were treated with thermolysin (+) to remove nonspecifically bound protein. Chloroplasts were also fractionated into soluble (S) and membrane (M) fractions, including envelope and thylakoid; an equal amount of the membrane fraction as in M was alkaline-treated (MA) to remove peripheral membrane proteins indicating Z-ISO is a membrane integral protein. C. Z-ISO is in a ~480 kD complex in chloroplasts. After *in vitro* import of radiolabeled Z-ISO, chloroplasts were treated with 0.5% Triton X-100 and protein complexes were separated on a Blue Native gel (Shumskaya and Wurtzel unp.).
Z-ISO contains heme iron. A. Cell pellets expressing MBP::Z-ISO were brown colored compared with pellets expressing MBP only. B. MBP::Z-ISO protein extracts were brown suggesting presence of a cofactor. C. MBP::Z-ISO was cleaved with TEV protease to release Z-ISO and MBP which were separated by SDS-PAGE and either stained for heme (left) or by Coomassie protein. Only individual bands are shown. D. Pyridine hemochrome assay shows heme b in Z-ISO. UV-visible absorption spectrum from a pyridine hemochrome assay of Ni-affinity purified MBP::Z-ISO protein extract (Blue: resting; Red: reduced). Bold numbers refer to peaks from the reduced heme.
Fig. 2.6. Spectral changes associated with the Z-ISO heme. A. Z-ISO in MBP::Z-ISO contains heme b that is largely ferric in the resting state of the enzyme. In the reduced state, the heme quantitatively binds CO (>90%). A. The UV-Vis spectrum of Z-ISO shows a shift when comparing the oxidized ("as-purified") and reduced forms of Z-ISO as well as when CO is added to enzyme with a dithionite-reduced heme. The spectral shift indicates displacement of the axial ligand in Z-ISO with CO. Absolute reduced spectrum: a band max = 559 nm. Soret (g) max = 426 nm; a /g = 10.7. B. The inset shows the 500-600 nm range for the dithionite-reduced sample. Two difference spectra for Z-ISO are shown. In the “reduced” minus “oxidized” spectrum, the graph was obtained by subtracting the UV-Vis spectrum of the dithionite-reduced enzyme from the spectrum of the enzyme “as-purified”. In the CO difference spectrum, the graph was obtained by subtracting the UV-Vis spectrum of the dithionite-reduced enzyme from the spectrum of the enzyme which was dithionite-reduced and then treated with CO.
Fig. 2.7. Binding of cyanide to Z-ISO. MBP::Z-ISO, 75.46 KDa (1.58 mg/ ml, 21 µM) , was incubated with KCN at a final concentration of 2 mM. UV visible spectra were recorded before and immediately after addition of KCN. Addition of cyanide caused a shift in the Soret band when the protein was in the oxidized state, indicating displacement and/or binding in place of an axial ligand in Z-ISO. The inset shows the difference spectrum, “oxidized + CN⁻” and “oxidized”.
Fig. 2.8. EPR spectra of Z-ISO. A. EPR spectrum of “as-purified” Z-ISO. B. A zoom-in view of the low-spin region of (A). C. EPR spectrum of dithionite-reduced Z-ISO. This sample is EPR-silent. D. EPR spectrum of reduced Z-ISO further treated with NO. The inset shows a zoom-in view of the nitrosyl adduct of Z-ISO.
**Fig. 2.9. Evidence for redox-dependent changes in ligand coordination.** MCD spectrum of Fe(III) Z-ISO (oxidized) is compared with a 50/50 mixture of Cyt. b₅ (bis-His) and Im-bound P450CAM (His-Cys). The low-spin heme (s) in ferric [Fe(III)] Z-ISO appear to have two sets of ligand pairs (His/His and His/Cys) in a ~1:1 ratio, either as a single species (with a common His) or as two separate heme centers. To distinguish between these possibilities, the MCD spectrum of ferrous [Fe(II)] Z-ISO (reduced) was compared with spectra of mono and bis Im-bound H93G Mb which shows that Z-ISO has only one heme center with alternate bis-His and His-Cys ligation to Fe(III) heme and bis-His to Fe(II) heme.
**Fig. 2.10. Functional testing of Z-ISO mutants.** Mutant genes were introduced into *E. coli* cells accumulating the Z-ISO substrate. Cells with empty vector also accumulate a small amount of product. Therefore enzyme activity is judged by the increase over this background level. Star indicates active enzyme. Carotenoids were extracted from the bacteria harboring the various enzyme variants and subjected to HPLC analysis to quantify the ratio of product ("di-cis" is 9,9'-di-cis-z-carotene) to substrate ("tri-cis" is 9,15,9'-tri-cis-ζ-carotene). At least three replicates were used for each construct.
FIG. 2.11. UV-Vis spectra of Z-ISO variants. Z-ISO::MBP fusions carrying H150A, H266A, or C263A were compared to wild type under reduced and “as-purified” conditions. Spectra for extracted proteins were normalized for absorbance at 280 nm.
Fig. 2.12. Ligand rearrangement demonstrated for the heme of Z-ISO A. Proximity of alternate ligands as in the Z-ISO homology model predicts feasibility of distal ligand switching between H266 and C263 where the heme proximal ligand is H150. B. The scheme integrates data showing binding by CO, NO, and CN⁻.
Fig. 2.13. **Model for Z-ISO isomerization.** The Fe(II) in the Z-ISO heme coordinates its electrons with the delocalized π electrons (shaded) of the 15-15’ cis C=C double bond of 9, 15, 9’ ζ-carotene. The resulting carbon-carbon bond becomes single bond in character and therefore is able to rotate to the thermodynamically favorable trans orientation.
Supplementary Fig. 2.1. MCD and UV-Vis calibration spectra for low spin (ls, Cyt. b5)/high spin (hs, Mb) states of ferric [Fe(III)] heme proteins. From this figure, it is estimated that Z-ISO contains >80% low spin heme.
Supplementary Fig. 2.2. Immunodetection of Z-ISO and Z-ISO variants (without transit peptide) expressed in *E. coli* cells harboring the pACCRT-EBP vector which confers accumulation of Z-ISO substrate. The control sample is from cells containing the pACCRT-EBP vector alone. Upper panel: Western blot analysis using anti Z-ISO. Lower panel: Coomassie stain for protein samples used for immunodetection.
Table 2.1. Summary of the EPR parameters for the iron species present in as-isolated Z-ISO

<table>
<thead>
<tr>
<th>Iron species</th>
<th>Ligands</th>
<th>( g ) factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-spin heme</td>
<td>His</td>
<td>5.8, 2.0</td>
</tr>
<tr>
<td>Low-spin heme #1</td>
<td>bis-His</td>
<td>2.98, 2.24(^a), 1.42</td>
</tr>
<tr>
<td>Low-spin heme #2</td>
<td>His-Cys</td>
<td>2.54, 2.24(^a), 1.86</td>
</tr>
<tr>
<td>Low-spin heme #3</td>
<td>His-Cys</td>
<td>2.50, 2.24(^a), 1.86</td>
</tr>
<tr>
<td>Low-spin heme #4</td>
<td>His-Cys</td>
<td>2.43, 2.24(^a), 1.92</td>
</tr>
<tr>
<td>Nonheme iron(^b)</td>
<td>N/D</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\(^a\)Multiple sets of overlapping signals
\(^b\)A minor species likely derived from non-specific iron binding
N/D: not determined
CHAPTER 3

Additional molecular and biochemical studies on Z-ISO

3.1 Introduction

In this chapter, results from other experiments related to those presented in chapter 2 are included as foundation for further research on Z-ISO. Evidence was provided on which residues in Z-ISO likely bind the heme and that the heme is linked to catalysis (Chapter 2). However, whether other close or remote locations in the protein are essential for Z-ISO function remains to be investigated (i.e. disordered domains required for putative protein-protein interactions or protein regulation, phosphorylation sites, residues involved in substrate binding). Based on previous sequence alignments (Chen et al., 2010), topology and disorder domain predictions, we generated and tested a series of Z-ISO truncations for isomerization activity.

The biochemical studies (Chapter 2) raise the possibility that the heme in Z-ISO may transiently coordinate the 15-15’ cis double bond of 9,15,9’ tri-cis-ζ-carotene to facilitate isomerization. Trapping an interaction between co-factor and substrate would be possible. To explore this aspect, purified protein (oxidized and reduced) was incubated with substrate and a UV spectrum was obtained.

Finally, since the presence of non-heme iron in purified Z-ISO remains to be understood, some functional data on other conserved residues that could potentially bind iron are also included.
3.2 Results

3.2.1 Analysis of truncated Z-ISO proteins

Z-ISO has a predicted transit peptide cleavage site of ~46 residues (Chapter 2) containing a first disordered domain from aa 14 to 38 as predicted by GLOBPLOT2 (Linding et al., 2003) (http://globplot.embl.de). A second and smaller disordered domain (disordered domain II) from residue 62 to 78 was also predicted in the N terminus. A truncated Z-ISO version where the first 46 aa were removed demonstrated that the transit sequence is not required for function (Fig. 3.1).

Further, five truncated versions of maize B73 ZmZ-ISO: Z-ISO.77_366 (Z-ISO T1), Z-ISO.136_366 (Z-ISO T2), Z-ISO.47_359 (Z-ISO T3), Z-ISO.77_359 (Z-ISO T4) and Z-ISO.136_359 (Z-ISO T5) (Table 3.3) were constructed to ascertain their ability to catalyze isomerisation of tri-cis-ζ-carotene to di-cis-ζ-carotene in E coli. Z-ISO fragments were amplified with N-terminal and C-terminal regions of the gene removed by targeting conserved regions identified across the gene family. Analysis of the truncated variants showed that Z-ISO T1, which lacks disordered domain II, was able to catalyze the isomerisation of the tri-cis-ζ-carotene to di-cis isomer as depicted in Figure 3.2. In contrast, results for Z-ISO T2, Z-ISO T3, Z-ISO T4 and Z-ISO T5 suggest that removal of the respective regions eliminates or reduces the ability of these Z-ISO constructs to catalyze the isomerisation in E coli (Fig. 3.2). These results also suggest that the C-terminus of Z-ISO is essential to its function. Deletion of the last seven amino acids (Z-ISO T5) from the C-terminal eliminated the Z-ISO function in E coli (Fig. 3.2).
3.2.2 Other residues tested for Z-ISO activity

As presented in Chapter 2, of the 12 His residues that might serve as potential heme or iron axial ligands, only 6 are absolutely conserved over a wide evolutionary span (e.g. comparing Z-ISO in plants, diatoms and algae). His residues were mutagenized to Ala and tested for isomerase function using complementation experiments in *E. coli* (Li et al., 2007; Chen et al., 2010). His-150 and His-266 were of our particular interest because, as showed above, modeling suggested that these amino acids are close enough to allow the binding of a prosthetic group like heme (Töro et al., 2009). H150A and H266A mutant variants were inactive as the main peak found by HPLC was 9,15,9'-tri-cis-ζ-carotene (Fig. 3.3). One aspartic acid (D-294) was also found to be required for Z-ISO function (Fig.3.3). In contrast, the other histidines residues, His47, His135, His191, His208, His241, His253, His285, His-286 and His-332, were not required for function as the main peak found by HPLC was 9,9'-di-cis-ζ-carotene (Fig. 3.4; Table 3.2). Several other residues were tested (Y223, Y301, Y340 and E136) but none of them appears to be required for function (Fig. 3.4). Other putative residues to be tested are listed in Table 3.3.

3.2.3 Binding of substrate

To test whether substrate can directly bind to the Z-ISO heme, purified protein was incubated with substrate and a UV spectrum was obtained. Substrate was added to both the oxidized and reduced forms of the enzymes, since both were shown to bind exogenous ligands. Under the conditions used here, no evidence for interaction between the heme in Z-ISO and the
substrate, ζ-carotene, could be found. Although the UV difference spectrum (plus vs. minus substrate) (Fig. 3.5) showed some differential peaks, these arose most likely from the substrate preparation itself or pipetting error. To verify this, the absorbance of substrate alone and Z-ISO alone were summed and used as control spectrum. If the heme of Z-ISO and the carotenoid are interacting under the experimental conditions used, the absorbance of the heme should be shifted from the control spectrum. However, the expected shift was not detected (Fig 3.6).

**3.3 Discussion**

Results indicated that the N terminal of Z-ISO protein contains two predicted disordered domains that were not required for function in *E. coli*. Disordered domains have been found to play a role in cell signaling, recognition, nucleic acid and protein-protein interactions (Chen et al., 2006). Thus, in the case of Z-ISO, disordered domains might have a yet to be identified role in plant chloroplasts. Interestingly, a short fragment of the protein at the C terminal (7 aa) was essential for Z-ISO function (e.g activity or proper folding). This finding is in agreement with the lack of isomerization activity of a shorter *Arabidopsis* Z-ISO transcript (ZISO1.2) which encodes a nonfunctional protein with one less TM domain (Chen et al., 2010).

With respect to the protein-substrate binding experiments, there are various possibilities to explain the absence of a detectable interaction. The overlapping spectroscopic signatures of the heme and the substrate could make this type of experiments difficult. Alternatively, solubilizing Z-ISO and ζ-carotene in DDM micelles, might not be the best strategy for enzyme-
substrate binding experiments. Also, the expected interaction might occur just too fast to be detected with the technology used here.

Despite the advances provided in this dissertation with regard to the role of heme in Z-ISO function, it is still unknown whether non-heme iron, present in purified MBP::Z-ISO, plays an accessory role in Z-ISO activity. Since mutation of Asp294 appears to block activity, the question therefore arises whether Asp294 in Z-ISO is a residue required for iron binding or for another unidentified function (i.e. protein folding, substrate binding). Further experiments are required to clarify the role of Asp294 in Z-ISO function. Other residues that potentially could bind iron are listed in Table 3.2.

Theoretically, non-heme iron could be providing electrons to the heme center or being necessary for another yet to be discovered function. These ideas should be tested in the context of protein-protein interactions (i.e. PDS/Z-ISO and Z-ISO/ZDS). Characterization of these interactions and enzymatic steps around Z-ISO will led to comprehension of the mechanisms for the synthesis of ζ-carotene.

3.4 Methods

3.4.1 Z-ISO truncations

Five truncated versions of maize B73 ZmZ-ISO: Z-ISO.77_366 (Z-ISO T1, #610), Z-ISO.136_366 (Z-ISO T2, #611), Z-ISO.47_359 (Z-ISO T3, #612), Z-ISO.77_359 (Z-ISO T4, #613) and Z-ISO.136_359 (Z-ISO T5, #614) were constructed. Z-ISO fragments were amplified
with N-terminal and C-terminal regions of the gene removed by targeting conserved regions identified across the gene family. Primers were designed with BamHI and SalI sites respectively on the forward and reverse primers. Primers were as follows: Z-ISO T1; sense CGGGATCCTCTCGTGGGTGAGGATTCGCTGGTTC and antisense GCGTCGACCTACCAGGGAAGTTGGTAGCT, Z-ISO T2; sense T2CGGGATCCTGAGGTTGTTATGTTGCTCCTACC and antisense GCGTCGACCTACCAGGGAAGTTGGTAGCT, Z-ISO T3; sense CGGGATCCTCAGGCTCCTCGGCCCCTCGGCT CGGGATCCTCTCGTGGGTGAGGATTCGCTGGTTC and antisense GCGTCGACCTGCCATCAATGGATGAGC, Z-ISO T4; sense CGGGATCCTCAGGCTCCTCGGCCCCTCGGCT CGGGATCCTCTCGTGGGTGAGGATTCGCTGGTTC and antisense GCGTCGACCTGCCATCAATGGATGAGC, Z-ISO T5; sense CGGGATCCTGAGGTTATGTTGCTCCTACC and antisense GCGTCGACCTGCCATCAATGGATGAGC.

PCR fragments were amplified using the Herculase II polymerase system, A-tailed and cloned into the pGEMT-Easy vector (Promega) for sequence confirmation. Fragments were then released by digestion with BamHI and SalI restriction enzymes and gel purified. The fragments were ligated between the BamHI and SalI sites in the pCOLA-Duet1 vector and transformed into E. coli. Plasmid DNA was isolated and confirmed through restriction digestion and agarose gel electrophoresis.

### 3.4.2 Site directed mutagenesis and functional complementation

pCola Zm Z-ISO NTP (#579) was used as template to perform substitutions of conserved residues to Ala as explained before (see methods section in chapter 2: *Site directed mutagenesis*

3.4.3 Binding of substrate

3.4.3.1 Substrate preparation. 9,15,9'-tri-cis-ζ-carotene (“tri”) was extracted from *E. coli* containing cultures harboring pACCRT-EBP (#150) and grown in LB with the same conditions explained above in the in vitro assay section. 200 mL of cell culture were centrifuged at 2,588 x g and pellet s were resuspended in a total of 20 mL of methanol, distributed in 4 falcon tubes with equal volumes of extract (5 mL) and sonicated on ice twice, 30 sec each, 60% power with a Vibra Cell VC600 sonicator equipped with a tapered 3 mm microtip (Sonics & Materials Inc, Connecticut, USA). Extracts were centrifuged at 2,588 x g for 10 min and supernants were transferred to 15 mL Falcon tubes and evaporated under nitrogen gas in the dark. Dried samples were resolubilized in 500 µL of methanol, transferred to 1,5 mL eppendorf tubes, frozen at 80°C for 1 h and centrifuged at 15,871 x g at 4°C. Extractions were then combined. Further, 1,75
mL (35.5 μM, estimated by spectroscopy using the molar extinction coefficient for ζ-carotene ($\varepsilon_{400} = 138,000$) of the extract was dried under N$_2$ in the dark. The dried sample was solubilized by vortexing in 1 mL of buffer (50 mM NaCl, 10% glycerol, 1% DDM and 20 mM Tris pH 7.6) resulting in a preparation of 62 μM substrate.

3.4.3.2 Protein-substrate binding. Substrate binding was evaluated by preparing three samples containing either, protein, protein plus substrate or a control containing substrate only. The control sample was prepared by mixing 120 μL (62 μM) of substrate with 380 μL of protein buffer (20 mM NaCl, 20 mM Tris (pH 7.6), 5% glycerol, 0.02% DDM, and 0.1 mM DTT), without protein. The same volumes were used to prepare a sample containing substrate buffer (120 μL; 50 mM NaCl, 10% glycerol, 1% DDM and 20 mM Tris pH 7.6 ) (no substrate) and Z-ISO protein (380 μL, 7.8 μM, resting, oxidized) and a sample containing substrate (120 μL, 62 μM) and Z-ISO protein (380 μL, 7.8 μM, resting, oxidized). The final substrate:protein ratio was 2.5: 1. The UV-visible spectrum was obtained using 500 μL samples. The spectrum of the sample containing substrate and protein was subtracted from the spectrum of the sample containing only substrate. The resulted spectrum was then subtracted from the spectrum of the sample containing only protein. The same experiment, with the same amounts and volumes for substrate and protein, was done to test binding with dithionite-educed protein by adding 1 mg dithionite as a powder to all samples before adding substrate.
Figure 3.1. Z-ISO transit peptide analysis. A. a truncated version of Z-ISO without its predicted transit peptide was generated. The truncated version was from amino acid 46 to 366. B. a chromatogram from HPLC for the functional analysis of the transit peptide. Empty: *E. coli* encoding bacterial GGPPS, PSY, and maize PDS accumulate 9,15,9’-tri-cis-ζ-carotene used as control. TP Zm Z-ISO: “empty” plus Z-ISO 1 to 366 including its transit peptide. Zm Z-ISO: “empty” plus Z-ISO 47 to 366 without its transit peptide.
Figure 3.2. Functional complementation of Z-ISO truncated versions (Beltrán, Dwamena and Wurtzel, unpub.). A. Schematic representation of deletion mutant Z-ISO proteins analyzed. B: chromatograms from HPLC for the functional analysis of the truncated versions described in A. Only Z-ISO 77-366 was functional as the mean peak accumulated was 9,9’-di-cis-ζ-carotene (Di) while the other truncations were non functional as the mean peak accumulated was 9,15,9’-tri-cis-ζ-carotene (Tri).
Figure 3.3. Functional testing of Z-ISO mutants. A. Independent substitutions of two conserved histidines residues, His-150 and His-266, and one conserved aspartic acid Asp-294, to alanine, block Z-ISO activity as tested by functional complementation in *E. coli* cells. Mutant genes were introduced into *E. coli* cells accumulating the Z-ISO substrate. Carotenoids were extracted from the bacteria harboring the various enzyme variants and subjected to HPLC analysis to quantify the ratio of product ("di-*cis*" is 9,9’ *di-cis*-ζ-carotene) to substrate ("tri-*cis*" is 9,15,9’ *tri-cis*-ζ-carotene). B. At least three replicates were used for each construct.
Figure 3.4. Functional testing of other conserved residues. Other residues tested by functional complementation in *E. coli* were not essential for Z-ISO activity as the mean peak accumulated was 9,9'-di-cis-ζ-carotene. (Sequences for E80A and E231A need to be confirmed).
3.5. Substrate binding to the Z-ISO heme. Enzyme as isolated (“oxidized”) (panel A) or reduced with dithionite (“reduced”) (panel B) were treated plus or minus the Z-ISO substrate as used in the in vitro reaction. Immediately after mixing, the UV-Vis spectrum was recorded. The difference spectra in the insets reveal that these peaks are most likely from substrate alone. The experiment was done with 19.5 μM substrate and 7.8 μM protein for a substrate/protein molar ratio of 2.5:1.
3.6 Binding of substrate. A. Binding of substrate with oxidized Z-ISO. The absorbance of substrate alone and Z-ISO alone were summed and used as a spectrum control. The spectrum of the mixture (substrate and MBP::Z-ISO) did not differ from the spectrum control. B. Binding of substrate with reduced Z-ISO. As in A, the absorbance for the mixture did not differ from the absorbance of the spectrum control. C. The absorbance difference between reduced Z-ISO and the sum of individual absorbances (substrate and Z-ISO) shows some minor amplitude differences that probably result from pipetting error (Hosler, Beltrán & Wurtzel, unpub). The experiment was done with 19.5 μM substrate and 7.8 μM protein for a substrate/protein molar ratio of 2.5:1. The experiment, then, indicates that under these conditions and these substrate concentrations, the substrate is not altering the heme environment.
<table>
<thead>
<tr>
<th>Truncation</th>
<th>Residues</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>77_366</td>
<td>Functional</td>
</tr>
<tr>
<td>T2</td>
<td>136_366</td>
<td>Nonfunctional</td>
</tr>
<tr>
<td>T3</td>
<td>47_359</td>
<td>Nonfunctional</td>
</tr>
<tr>
<td>T4</td>
<td>77_359</td>
<td>Nonfunctional</td>
</tr>
<tr>
<td>T5</td>
<td>136_359</td>
<td>Nonfunctional</td>
</tr>
</tbody>
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**Table 3.1.** Truncated versions of *Zm* Z-ISO tested by the *E.coli* complementation system.
<table>
<thead>
<tr>
<th>Amino acid Substitution</th>
<th>Putative for</th>
<th>Required for Function?</th>
<th>Conserved?</th>
</tr>
</thead>
<tbody>
<tr>
<td>H150A</td>
<td>heme, non-heme iron</td>
<td>Yes</td>
<td>Across species</td>
</tr>
<tr>
<td>H191A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across species</td>
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<tr>
<td>H208A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across plants</td>
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<tr>
<td>H241A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across species (except Z-ISO_Pm)</td>
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<tr>
<td>H253A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across species</td>
</tr>
<tr>
<td>H354 A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across plants</td>
</tr>
<tr>
<td>H266A</td>
<td>heme, non-heme iron</td>
<td>Yes</td>
<td>Across species</td>
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<tr>
<td>H285A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across species</td>
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<td>H286A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across species</td>
</tr>
<tr>
<td>Y223A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across species (except Z-ISO_Se)</td>
</tr>
<tr>
<td>Y301A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across plants</td>
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<td>Y340A</td>
<td>heme, non-heme iron</td>
<td>No</td>
<td>Across plants</td>
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<td>E80A</td>
<td>non-heme iron</td>
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<td>E136 A</td>
<td>non-heme iron</td>
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<td>D294A</td>
<td>non-heme iron</td>
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<td>Across species</td>
</tr>
<tr>
<td>C263A</td>
<td>Heme, non-heme iron</td>
<td>No</td>
<td>Across species (except Z-ISO_Tp)</td>
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</table>

**Table 3.2.** List of Z-ISO residues mutagenized and tested for function.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Putative for</th>
<th>Conserved?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y193</td>
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<td>Across plants</td>
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<tr>
<td>E231</td>
<td>heme, non-heme iron</td>
<td>Across species</td>
</tr>
<tr>
<td>E244</td>
<td>heme, non-heme iron</td>
<td>Across species</td>
</tr>
<tr>
<td>D330</td>
<td>heme, non-heme iron</td>
<td>Across plants</td>
</tr>
<tr>
<td>Y351</td>
<td>heme, non-heme iron</td>
<td>Across species</td>
</tr>
</tbody>
</table>

**Table 3.3.** List of Z-ISO residues to be tested in future projects.
CHAPTER 4

Summary and future perspectives

4.1 Summary

It was previously demonstrated that Z-ISO is required for isomerization of a 15-cis carbon-carbon double bond in ζ-carotene in *E. coli* cells producing the Z-ISO substrate 9,15,9’ tri-cis-ζ-carotene (Chen et al., 2010). In that *E. coli* system the addition of a Z-ISO gene led to conversion of 9,15,9’ tri-cis-ζ-carotene into 9,9’-di-cis-ζ-carotene. However, this Z-ISO activity occurred in the presence of several upstream carotenoid biosynthetic enzymes needed to produce the Z-ISO substrate. Therefore, it was unclear whether Z-ISO isomerization was a result of its inherent catalytic activity and how the isomerization might be catalyzed.

In this study, bioinformatic approaches were applied to get further insight into Z-ISO location and function. Topology-prediction programs predicted 7 TM domains in maize Z-ISO which suggested that Z-ISO is an integral membrane protein. *In vitro* import assays (Shumzakaya and Wutzel unpl.) further confirmed that Z-ISO localizes to the chloroplast membrane.

Further, homology modeling tools were also applied to look for structural homologies. Meta Server modeled Z-ISO onto an integral membrane protein, the diheme cytochrome b subunit of quinol:fumarate oxidoreductase. Although neither NnrU nor Z-ISO are annotated as metalloproteins, homology modeling predicted that Z-ISO contains heme or nonheme iron. We therefore tested potential residues that function as ligands of nonheme iron or heme. Two
conserved histidines (H150, H266) were required for activity, as well as one aspartic acid residue (D294). Moreover, predicted locations of H150 and H266 based on the Z-ISO structure model are consistent with coordination of a common cofactor.

Finally, Z-ISO was biochemically characterized and its activity was investigated using both in vivo and in vitro assays. The in vitro assay developed here demonstrated that Z-ISO is an enzyme on its own under reducing conditions. Extensive biochemical characterization demonstrated that Z-ISO is an integral membrane metalloprotein containing heme b, which appears to switch one of its axial ligands depending upon the redox state of the heme. Our work points out important Z-ISO features which led us to conclude that Z-ISO requires heme b for activity.

4.2 Future Perspectives

While this research offered insights into the features that allow Z-ISO to be an isomerase in vitro and in E. coli, its regulation and topology with respect to other enzymes in plastids have yet to be elucidated. If Z-ISO is an integral membrane protein, it is reasonable to postulate protein-protein interactions with upstream and downstream enzymes in the pathway that are peripherally bound to the chloroplast membrane (i.e. PDS, ZDS). Understanding the mechanisms controlling the formation of these protein complexes will allow predictive metabolic engineering of the carotenoid pathway for enhanced provitamin A content. Bimolecular fluorescence complementation (BiFC) and in vitro studies are required to clarify this point.
In a functional perspective, albeit a low range of activity, Z-ISO performs the expected catalytic activity \textit{in vitro} under reducing conditions and in the absence of other enzymes from the pathway. However, the redox modulator for Z-ISO \textit{in vivo} remains to be identified. It is known that photosynthesis drives thioredoxin reduction needed for electron transfer to target proteins (Serrato et al., 2004). In \textit{Arabidopsis}, thioredoxin genes are highly co-expressed with Z-ISO. In cyanobacteria, Z-ISO is also linked to a thioredoxin gene, which suggests related function (Chen et al., 2010). Therefore, thioredoxin could potentially supply electrons to reduce heme iron in Z-ISO. Further experiments are necessary to test this hypothesis.

In the same vein, it is important to note that besides heme, our results also showed the presence of non-heme iron in Z-ISO. At this point is still unclear whether non-heme iron has a functional connection (i.e. providing electrons to the heme) with the heme center. However, the evidence presented here led to the conclusion that heme b is required for Z-ISO function.

Interestingly, 9,9'-di-cis-\(\zeta\)-carotene; the product of Z-ISO, is involved in the generation of a yet to be indentified molecule that controls leaf development and the expression of chloroplast and nuclear genes in \textit{Arabidopsis} (Avendano-Vazquez et al., 2014). Therefore, finding out how Z-ISO is regulated to generate \(\zeta\)-carotene would be important to understand how the carotenoid pathway contributes to the signaling processes controlling leaf development.

Given that Z-ISO is a heme binding protein that binds NO, as we show here, it is reasonable to suggest that NO could potentially be a regulator for Z-ISO activity \textit{in vivo}. NO is
produced in the chloroplast and studies suggest it is involved in carotenoid biosynthesis regulation in plants (Chang et al., 2013). Finding out if, or how, NO regulates carotenoid biosynthesis appears to be an interesting topic of research. Further studies on the activity of Z-ISO in plants subjected to different stresses are required to test this hypothesis.

Although heme proteins such Z-ISO can be extensively studied using spectroscopic techniques, further efforts on protein crystallization and structure determination will be necessary to fully understand its mechanism of function. A crystal structure will better guide mutagenesis experiments to elucidate fine details on catalytic function. In this study, we used DDM to solubilize Z-ISO from *E. coli* membranes. However, it might be worth to perform a comprehensive screening for selecting the most suitable detergent for protein folding and crystallization efforts. The challenge will be to obtain a crystal structure with both, the heme and the substrate, bound to Z-ISO. It is expected that all the methods developed in this dissertation for the study of Z-ISO will be useful for future research.

Finally, it should be noted that Z-ISO is a single copy gene in plants and it is unknown whether Z-ISO natural variation drives carotenoid accumulation changes in plant edible tissues among different crops. Understanding of allelic differences can be targeted in breeding projects to produce more nutrition crops (Wurtzel et al., 2012). For example, natural variation in lycopene epsilon cyclase (lcyE) explain differences in provitamin A content in maize (Harjes et al., 2008). In this context, the question arises as to how re-engineering Z-ISO will help to enhance metabolic flux towards the formation of pro-vitamin A carotenoids. Alternatively, since early
studies suggest that plants with insufficient Z-ISO grow poorly under the stress of fluctuating temperature, Z-ISO could potentially be used towards the generation of crops better adapted to changing environments.
Appendix 1: Z-ISO expression and purification protocol

Construct: pMCSG9 Z-ISO E2 (# 582) in C43 (DE3) cells.

1. Set overnight cultures (10 mL per L) in 2X YT medium (2x yeast extract and tryptone; 1% yeast extract, 1.6% tryptone and 0.5% NaCl) containing Ampicillin 50 mg/L.
2. Add 10 mL of overnight culture per liter of 2X YT medium. Incubate at 200 r.p.m and 37°C until an O.D. of 0.6 (typically ~2 h).
3. Induce with 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG, Gold Biotechnology) and incubate for 16 h at 28°C.
4. Centrifuge cells at 2,600 x g (3,500 r.p.m, Eppendorf centrifuge 5810 R) for 15 minutes at 4 °C and freeze (-80 °C) pellets until use.
5. Resuspend at a ratio of 50 mL per 8 g of cell pellets, (~ 40 mL per liter of initial culture) in Resuspension Buffer (50 mM Tris pH 7.6, 300 mM NaCl, and 5% glycerol) containing (freshly added) 0.5 mM dithiothreitol (DTT, VWR International), 4 μL/25 mL benzonase (Sigma-Aldrich), 60 mg/50 mL of 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Bio-Research products) and 0.15 mg/mL of lysozyme from chicken egg white (USB Corporation).
6. Sonicate on ice (5 times, 30 sec each, 60% power, with 1 min between sonication) using a Vibra Cell VC600 sonicator equipped with a 3 mm tapered microtip (Sonics & Materials Inc, Connecticut, USA).
7. Centrifuge at ~9800 x g (11,000 r.p.m, in a Type 60 Ti rotor) for 15 minutes at 4 °C using Polycarbonate tubes (25 x 89 mm, Beckman coulter). Save lysates.
8. Centrifuge lysates at ~83,000 x g (32,000 r.p.m, in a Type 60 Ti rotor) for 1 h at 4 °C using Polycarbonate tubes (25 x 89 mm, Beckman Coulter).
9. Resuspend pellets in Resuspension Buffer at a ratio of 8 mL per liter of initial cell culture, following by sonication as described above or until pellets are dissolved.
10. Increase the volume to a total of 40 mL of Resuspension Buffer per liter of starting culture. Add n-Dodecyl β-D-maltoside (DDM, Antragrace) added as powder to a final concentration of 1.5 % and rotate samples end over end at 4°C for 15 min.
11. Incubate lysates overnight with equilibrated Ni-NTA containing resin from Qiagen
   [Equilibrate resin twice with Resuspension Buffer by centrifugation at 500 x g (~ 1500 r.p.m in a effendorff caentrifuge 5424 R) at a ratio of 300 μL resin per 40 mL of lysate in Falcon tubes.]
12. Centrifuge Falcon tubes at 500 x g (~ 1500 r.p.m. Eppendorf centrifuge 5810 R). Careful discard the supernat and transfer the resin pellet to a 5 mL polypropylene column (Qiagen).
13. Wash with 5 resin volumes of ATP Wash Buffer (40 mM Tris pH 7.6, 200 mM NaCl, 5% glycerol, and 5 mM MgCl₂), containing freshly added final concentrations of 5 mM ATP
(Fisher Scientific), 0.1 mM DTT and 0.05% DDM for 30 min (column under gentle rotation at 4 °C)

14. Wash (5 resin volumes) with Wash Buffer (40 mM Tris pH 7.6, 400 mM NaCl, 5% glycerol) containing freshly added final concentrations of 0.1 mM DTT, 0.05% DDM and 30 mM Histidine (Sigma-Aldrich) for 5 min (column under gentle rotation at 4 °C).

15. Elute with Elution Buffer (25 mM Tris pH 7.6, 200 mM NaCl, 200 mM Histidine and 5% glycerol) containing freshly added final concentrations of 0.1 mM DTT and 0.05% DDM at a ratio of 1 mL Elution Buffer per liter of initial culture.

16. Dialyze the sample overnight using a Slide-A-Lyzer Dialysis cassette G2 20 K membrane (Thermo-scientific, IL, USA) against 1000-fold volume of buffer containing 20 mM NaCl, 20 mM Tris (pH 7.6), 5% glycerol, 0.02% DDM, and 0.1 mM DTT at 4 °C.

17. Quantify protein using absorbance at 280 nm in nanodrop (nanodrop 1000 spectrophotometer, Thermo scientific). Set the nanodrop at 75.46 KDa and E & MW 130.75. The yield is ~ 0.8-1.0 mg /L. Protein is isolated at ~90% purity.

18. For metal analysis, include 1 mM EDTA (Sigma-Aldrich) in the dialysis buffer and dialyze three times, 3 h each.

19. If needed, concentrate the sample using microconcentrators (Microcon microconcentraros 100 K, Amicon, Inc., Beverly, MA USA) and centrifugation at 2500 r.p.m (~ 600 x g) 3 min each, mixing the sample by pipeting.

20. For EPR, MCD, UV-visible and ICP-OES protein is storage at -20 °C, as isolated. For in vitro assays, store the protein at -20 °C in buffer containing 20 mM NaCl, 20 mM Tris (pH 7.6), 40 % glycerol, 0.02% DDM, and 0.1 mM DTT. Quantify again using nanodrop. For EPR use 300 μL at 15.1 mg/mL (~200 µM) for a total of 4.55 mg. For MCD use 1 mL at 1.57 mg/mL (~ 20 µM) for a total of 1.57 mg. For ICP-OES use 1 mL at 1 mg/ mL for atotal of 1 mg. (To avoid precipitation, add salts and detergent first, mix before adding glycerol)
<table>
<thead>
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<th>Reagents</th>
<th>Source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropyl-beta-D-thiogalactopyranoside (IPTG)</td>
<td>Gold Biotechnology</td>
<td>12481C25</td>
</tr>
<tr>
<td>1,4-Dithiothreitol (DTT)</td>
<td>VWR International</td>
<td>VW1506-02</td>
</tr>
<tr>
<td>4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride-HCl (AEBSF).</td>
<td>Bio-Research products</td>
<td>50-401-001</td>
</tr>
<tr>
<td>n-Dodecyl β-D-maltoside (DDM)</td>
<td>Anatrace</td>
<td>D310</td>
</tr>
<tr>
<td>Adenosine triphosphate (ATP)</td>
<td>Fisher</td>
<td>987-65-5</td>
</tr>
<tr>
<td>Lysozyme from chicken egg white</td>
<td>USB, Corporation</td>
<td>18645</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Fisher</td>
<td>7647-14-5</td>
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<tr>
<td>Tris</td>
<td>Invitrogen</td>
<td>15504-020</td>
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<tr>
<td>Yeast extract</td>
<td>Fisher</td>
<td>BP1422-500</td>
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<tr>
<td>Tryptone</td>
<td>Fisher</td>
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<tr>
<td>Ni-NTA agarose</td>
<td>Qiagen</td>
<td>30210</td>
</tr>
<tr>
<td>Slide-A-Lyser dialysis cassettes G2 20K</td>
<td>Thermo-scientific</td>
<td>87735</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher (across organics brand)</td>
<td>AC15892-0010</td>
</tr>
<tr>
<td>Bottle, with Cap Assembly, Polycarbonate, 26.3 mL, 25 x 89 mm, 1 x 3-1/2</td>
<td>Beckman Coulter</td>
<td>355618</td>
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<td>Ampicillin</td>
<td>Sigma-aldrich</td>
<td>A0166-256</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma-aldrich</td>
<td>E-5134</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl2)</td>
<td>USB</td>
<td>18641</td>
</tr>
</tbody>
</table>

**Appendix Table 1.1.** List of reagents including source and catalog number.

General use buffers:

**1M Tris-HCl, 1L**
- Dissolve 121.1 g of Tris base in 800 ml of H2O.
- Adjust the pH to 7.6 by adding concentrated HCl (~ 60 mL).
- Adjust the volume of the solution to 1 liter with ddH2O. Sterilize by autoclaving (15 minutes)

**5M NaCl, 1L**
- Mix 292.2 g of NaCl with 900 ml of ddH2O by stirring.
- Add ddH2O until final volume is 1 L. Sterilize by autoclaving (15 minutes)

**1M MgCl2, 100 mL**
- Mix 20.33 g MgCl2 with 70 ml of ddH2O
- Add ddH2O until final volume is 100 mL. Sterilize by autoclaving (15 minutes)
Protein purification buffers for 2 L of initial culture.

**Resuspension buffer (200 mL)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock</th>
<th>Final volume 200 mL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH 7.6</td>
<td>1M</td>
<td>10 mL</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>5M</td>
<td>12 mL</td>
<td>300 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100%</td>
<td>10 mL</td>
<td>5 %</td>
</tr>
<tr>
<td>ddH2O</td>
<td></td>
<td>168 mL</td>
<td></td>
</tr>
</tbody>
</table>

Add fresh:
- 240 mg of AEBSF
- 32 µL of benzonase
- 100 µL of DTT, 1M
- 30 mg of lysozyme

**ATP buffer (3 mL)**

<table>
<thead>
<tr>
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<th>3 mL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH 7.6</td>
<td>1 M</td>
<td>120 µL</td>
<td>40 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 M</td>
<td>120 µL</td>
<td>200 mM</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1 M</td>
<td>15 µL</td>
<td>5 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100%</td>
<td>150 µL</td>
<td>5%</td>
</tr>
</tbody>
</table>

Add fresh:
- 8.25 mg of ATP
- 0.3 µL of DTT, 1M
- 15 µL of DDM, 10%
Adjust to 3 mL with ddH<sub>2</sub>O

**Wash buffer (5 mL)**

<table>
<thead>
<tr>
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<th>5 mL</th>
<th>Final concentration</th>
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<tr>
<td>Tris HCl pH 7.6</td>
<td>1M</td>
<td>0.2 mL</td>
<td>40 mM</td>
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<tr>
<td>NaCl</td>
<td>5M</td>
<td>0.4 mL</td>
<td>400 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100%</td>
<td>250 µL</td>
<td>5%</td>
</tr>
</tbody>
</table>

Add fresh:
- 0.5 µL of DTT 1M
- 25 µL of DDM, 10%
- 600 of µL of histidine 0.25 M pH 7.8
Adjust to 5 mL with ddH<sub>2</sub>O
### Elution buffer (5 mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock</th>
<th>Final volume 5 mL</th>
<th>Final concentration</th>
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</thead>
<tbody>
<tr>
<td>Tris HCl pH 7.6</td>
<td>1M</td>
<td>125 µL</td>
<td>25 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>5M</td>
<td>200 µL</td>
<td>200 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100%</td>
<td>250 µL</td>
<td>5 %</td>
</tr>
</tbody>
</table>

Add fresh:
- 0.5 µL of DTT 1M
- 25 µL of DDM, 10%
- 4 mL of histidine 0.25 M pH 7.8
Adjust to 5 mL with ddH₂O

### Dialysis buffer (2 L)

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock</th>
<th>Final volume 2 L</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH 7.6</td>
<td>1M</td>
<td>40 mL</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>5M</td>
<td>8 mL</td>
<td>20 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100%</td>
<td>100 mL</td>
<td>5 %</td>
</tr>
</tbody>
</table>

Add fresh:
- 0.4 g of DDM
- 0.2 mL of DTT 1M
Appendix 2: Z-ISO in vitro enzyme assay protocol

A. Substrate extraction

Construct: pACCRT-EBP (#150) in E. coli BL21 (DE3) cells.

1. Inoculate 400 mL of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing chloramphenicol (34 μg mL⁻¹) with 8 mL of overnight culture containing pACCRT-EBP (#150).
2. Grow cultures in the dark at 37 °C, at 160 rpm for 8 h before induction with 10 mM IPTG (Gold Biotechnology).
3. Incubate at 28 °C, at 100 rpm for 40 h and an additional 2 d without shaking.
4. Centrifuge cells at 2,588 x g (~ 3,500 r.p.m, Eppendorf centrifuge 5810 R) and save pellets.
5. Resuspend pellets in 40 mL of methanol (VWR, HPLC grade), distributed in 4 Falcon tubes with equal volumes of extract and sonicate twice on ice, 30 sec each at 60% power using a Vibra Cell VC600 sonicator equipped with a tapered 3 mm microtip (Sonics & Materials Inc, Connecticut, USA).
6. Centrifuge at 2,588 x g (~ 3,500, Eppendorf centrifuge 5810 R) for 10 min and transfer supernants to 15 mL Falcon tubes. Evaporate samples under Nitrogen gas in the dark.
7. Add 300 μL of methanol (VWR, HPLC grade) to each tube, transfer to 1.5 mL eppendorf tubes, incubate at 80°C for 1 h and centrifuge at 15,871 x g (~ 13000 r.p.m, Eppendorf centriufuge 5424R) at 4°C.
8. Combine extractions (~ 1.2 mL) and use 1 mL used to prepare liposomes.

B. Preparation of substrate-containing liposomes.

9. Mix 1 mL of substrate extract [58 μM, estimated by spectroscopy (mix 50 μl of the carotenoid extraction with 450 μl of methanol, take the absorbance at 400 nm and estimate the amount using the molar extinction coefficient for ζ-carotene; ε₄₀₀ = 138,000 and the dilution factor) with 35 μL of soybean L-α-Phosphatidylcholine (Sigma-Aldrich) (20 mg mL⁻¹ in methanol). Dry the mixture under N₂.
10. Prepare sonication buffer [25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Sigma-Aldrich] pH 7.8, 100 mM NaCl, 10% glycerol) and bubble for 1 min with N₂ gas. Add 800 μL to the dried carotenoid lipid mixture.

11. Sonicate on ice using a Vibra Cell VC600 sonicator equipped with a 3 mm tapered microtip (Sonics & Materials Inc, Connecticut, USA) for 1 min at intervals of 10 s at 20% power. Wait for 1 min between each sonication.
C. *In vitro* reactions (final volume of 400 μL).

1. Incubate protein (10 μM final concentration), as purified in Appendix 1, with 15 μL of AcTEV protease [150 units, Invitrogen (AcTEV is an improved version of Tobacco Etch Virus, TEV)] for 2 min at RT.
2. Add freshly prepared sodium dithionite (MW: 174.107 g/mol, sodium hydrosulfite, Sigma-Aldrich) final concentration of 10 mM.
3. Add 200 μL of substrate-containing liposomes, (for a final concentration of ~ 36 μM substrate).
4. Overlay with N₂ gas before capping. Incubate at 28 °C under continuous shaking at 130 rpm for 3 h in the dark. For negative control, assemble reactions with heat denatured (10 min at 100°C) MBP::Z-ISO E2 fusion.
5. Extract reactions by adding 1 mL of petroleum ether/diethyl ether 2:1 (v/v). Collect the organic phase (upper layer) and dry under N₂. Disolve in 150 μL methanol and use 100 μL for HPLC.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropyl-beta-D-thiogalactopyranoside (IPTG)</td>
<td>Gold Biotechnology</td>
<td>I2481C25</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Sigma-Aldrich</td>
<td>C-0378</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Fisher</td>
<td>7647-14-5</td>
</tr>
<tr>
<td>Methanol (HPLC grade)</td>
<td>VWR (OmniSolv brand)</td>
<td>EM-MX0488-1</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>Sigma-Aldrich</td>
<td>261734-1L</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>Alfa Aesar</td>
<td>38990</td>
</tr>
<tr>
<td>Soybean L-α-Phosphatidylcholine</td>
<td>Sigma-Aldrich</td>
<td>P7443-100MG</td>
</tr>
<tr>
<td>HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid)</td>
<td>Sigma-Aldrich</td>
<td>H3375-250G</td>
</tr>
<tr>
<td>Sodium dithionite</td>
<td>Sigma-Aldrich</td>
<td>157953-100g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Fisher</td>
<td>BP1422-500</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Fisher</td>
<td>BP1421-2</td>
</tr>
</tbody>
</table>

**Appendix Table 2.1.** List of reagents including source and catalog number
General use buffers:

**1M HEPES, 1 L**
-Dissolve 238.3 g HEPES in 800 mL of ddH2O
-Adjust the pH to 7.8 with 10 N NaOH
-Bring up the volume to 1 L with ddH2O

**Sonication buffer (2 mL)**

<table>
<thead>
<tr>
<th>Stock</th>
<th>Final volume 2 mL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES pH 7.8 1M</td>
<td>50 µL</td>
<td>25 mM</td>
</tr>
<tr>
<td>NaCl 5M</td>
<td>40 µL</td>
<td>100 mM</td>
</tr>
<tr>
<td>Glycerol 100%</td>
<td>200 µL</td>
<td>10%</td>
</tr>
</tbody>
</table>
Appendix 3: Separation of ζ-carotene isomers by HPLC

1. Use a HPLC system equipped with a 2695 separation module, 996 photodiode array detector (Waters), and Empower 2 software (Waters).
2. Verify that a C30 Develosil 5u RPAQUEOUS (250 x 4.6 mm) column from Phenomenex (Nomura Chemical Co. Ltd, Seto, Japan) is attached to the HPLC system. Turn on the detector lamp.
3. Check that solvent bottles are set as follows: Solvent A is water (Milli-Q), Solvent C is methyl-t-butyl-ether (VWR, BDH) and Solvent D is Methanol (VWR, HPLC grade).
4. Use the following mobile phase: 4 parts water (Milli-Q), 66 parts methanol (VWR, HPLC grade), and 30 parts methyl-t-butyl-ether (VWR, BDH) at a constant flow rate of 1 mL min⁻¹ for 80 min. (This method is saved as RQ-Method 1 in the Empower 2 software).
5. Prime the system (wet priming) at a 100% solvent composition for each solvent, 3 min, at a 7.5 mL/min flow rate.
6. In the running panel of the Empower software, set up a run as follows:
   - “condition column”, “RQ_method1”, 20 min.
   - Set run for 100 µL of each sample as “inject samples”, “RQ_method1”, 80 min.
   - Column wash as “condition column”, “95Water_5Methanol_Jesus”, 20 min, “condition column”, “100methanol_JESUS”, 20 min, and “condition column”
   - “Shutdown NarrowBore_JESUS”, 20 min.
7. Start run. Load 130 µL in 1.5 mL vials (National Scientific) with 150 µL inserts (National Scientific).
8. When the run is over turn off the detector lamp. On the Empower software check results at 400 nm. Select area peaks and check spectra (see Appendix Fig 3.1).

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
<th>Cat. number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP Blue cap with T/RR septa 100/PK, 1000/CS</td>
<td>National Scientific</td>
<td>C4000-51B</td>
</tr>
<tr>
<td>Polyspring inserts, 150uL, 100/Pk</td>
<td>National Scientific</td>
<td>C4012-530</td>
</tr>
<tr>
<td>Target DP vials, 1.5 mL</td>
<td>National Scientific</td>
<td>C4000-2W</td>
</tr>
</tbody>
</table>

Appendix Table 3.1. List of items for HPLC sample run.
Appendix Figure 3.1. HPLC analysis of ζ-carotene isomers extracted from *E. coli* cells. A, HPLC scans. B, Peak spectra. 9,15,9'-tri-cis-ζ-carotene (Tri); 9,9'-di-cis-ζ-carotene (Di).
Appendix 4: Generation of antibodies against Z-ISO

A. Antibody generation

To generate an antibody against Z-ISO, 2 mg of purified MBP Z-ISO E2 protein (#582) were digested with TEV protease to generate free Z-ISO. Samples were separated using the NuPAGE system (Invitrogen, Carlsbad, USA). Protein bands corresponding to Z-ISO were sliced out and shipped to Lampire Biological laboratories for rabbit immunization. Polyclonal antibodies against Z-ISO were generated in 2 rabbits identified as 190202 and 190203. The serum for each rabbit was collected at different times. Four serum samples at different dates were received: 07-25-13, 08-15-13, 08-22-13 and 09-23-13.

B. Western blot

For immunodetection protein samples were separated by electrophoresis using the NuPAGE system (Invitrogen). Reducing conditions in the samples were generated with DTT (100 mM). Proteins were transferred onto nitrocellulose membranes (Optitran; Whatman, Dassel, Germany) using an electrophoretic transfer cell (Criterion Blotter, Bio-Rad) at 20 V overnight, 4 °C using 1 X transfer buffer (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol). The membranes were then incubated in blocking buffer [1X Phosphate Buffered Saline buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$)], 3 % Bovine Serum Albumin (BSA, Fisher, cat. # BP9703-100) and 1 % Tween 20 for 1 h at RT, then with the rabbit anti-Z-ISO polyclonal antibody (for purified MBP::Z-ISO a 1:10000 dilution was used, for total protein extracts from E. coli a 1:2000 dilution was used) for 1 h at RT. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, cat. # 656120).
for 1 h at RT and washed with 1 X PBS (Phosphate buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$) buffer containing 1 % Tween 20 for 15 min followed by 4 additional washes of 5 min each. Immunoreactions were visualized with the Super Signal West Dura kit (Thermo Scientific, cat. # 37071). Fluorescent signals were captured using a G-box (Chemi XT4) from Syngene with Genesys V1.3.1.0 Software.

C. Testing production bleeds

Production bleeds from two rabbits (192002 & 192003) were used at different concentrations (1:2000 & 1:10000) for detecting two different amounts of Z-ISO protein (0.1 & 1 µg). Results suggest that the bleeds tested from both rabbits react with Z-ISO. Bleed #2 (08-15-13) from rabbit 192003 at either dilution gave the best result judging by band intensity and lower background (Appendix Fig. 4.1). In a second test (Appendix Fig. 4.2), bleed #4 (09-23-13) from rabbit 190203 gave the best results.
Appendix Figure 4.1. Testing rabbit bleeds for Z-ISO immunodetection. Two rabbit bleeds (192002 and 19203) from two different dates (08-15-13 and 08-22-13) were tested for Z-ISO immunodetection. Two different dilutions (1:2000 and 1:10000) were used for each bleed. All the bleeds tested were reactive against Z-ISO (0.1 and 1 μg). The best result was obtained with bleed 08-22-13 from rabbit 192003.
Appendix Figure 4.2. Testing rabbit bleeds (Date: 09-23-13) for Z-ISO immudetection. All the bleeds tested are reactive against Z-ISO. Rabbit 192003 bleed (Date: 09-23-13) gave the best results as judging from band intensity.
Appendix 5: A co-authored publication related to this dissertation

Synergistic Interactions between Carotene Ring Hydroxylases Drive Lutein Formation in Plant Carotenoid Biosynthesis

Rena F. Quinlan, Maria Shumskaya, Louis M.T. Bradbury, Jesus Beltran, Chunhui Ma, Edward J. Kennelly, and Elenaore T. Wurtzel

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Plant carotenoids play essential roles in photosynthesis, photoprotection, and as precursors to xanthophylls. The plastid-localized carotenoid biosynthetic pathway is mediated by well-defined nucleic-encoded enzymes. However, there is a major gap in understanding the nature of protein interactions and pathway complexes needed to mediate carotenogenesis. In this study, we focused on carotene ring hydroxylases, which are performed by two structurally distinct classes of enzymes, the P450 CYP97A and CYP97C hydroxylases and the nonheme diiron HYD enzymes. The CYP97A and HYD enzymes both function in the hydroxylation of β-rings in carotenoids, but we show that they are not functionally interchangeable. The formation of lutein, which involves hydroxylation of both β- and e-rings, was shown to require the cooperation of CYP97A and CYP97C enzymes. These enzymes were also demonstrated to interact in vitro and in vitro, as determined using bimolecular fluorescence complementation and a pull-down assay, respectively. We discuss the role of specific hydroxylase enzyme interactions in promoting pathway flux and preventing the formation of pathway dead ends. These findings will facilitate efforts to manipulate carotenoid content and composition for improving plant adaptation to climate change and/or for enhancing nutritionally important carotenoids in food crops.

Carotenoids are a large class of isoprenoid pigments synthesized by all photosynthetic organisms as well as some bacteria, fungi, and aphids (Cuttress et al., 2011). In plants, carotenoids serve essential roles in photosynthesis and photoprotection (Jahn and Holsworth, 2012) and are precursors to xanthophylls (Wurtzel et al., 2010). Plant-derived carotenoids also provide nutritional benefits to humans (von Lintig, 2010; Wurtzel et al., 2012).

The plastid-localized carotenoid biosynthetic pathway is mediated by well-defined nucleic-encoded enzymes.

The product of the first committed biosynthetic step, phytoene, is enzymatically converted into all-trans-lycopene, the major branch point precursor for downstream carotenoids (Fig. 1). The linear lycopene is enzymatically converted to carotenoids by the formation of an e-ring or β-ring at each end of lycopene. The rings differ only in the position of a double bond. Hydroxylation of the carotene rings is mediated by ring-specific hydroxylases and leads to xanthophylls such as lutein and zeaxanthin.

Although the individual biosynthetic enzymes are known, there is a gap in the fundamental understanding of complexes and protein interactions involved in mediating carotenogenesis. The pathway likely functions as a multienzyme complex(es) to facilitate metabolite channeling, as predicted by the absence of pathway intermediates and the presence of complexes containing carotenoid biosynthetic enzymes (Weis et al., 1977; Camara et al., 1982; Kreuz et al., 1982; Baldal et al., 1996; Bonk et al., 1997; Lopez et al., 2008). In this study, we examined an intriguing portion of the pathway, where hydroxylation of rings in carotenoids is catalyzed by two structurally distinct enzymes. P450 heme (CYP97) and nonheme diiron (HYD) enzymes (Sun et al., 1996; Tian and DellaPenna, 2001, 2004; Kim and DellaPenna, 2008; Quinlan et al., 2007). Hydroxylation of the two β-ionone rings in β-carotene leads to a formation of zeaxanthin, while hydroxylation of the one β-ring and one e-ring in α-carotene leads to lutein. Hydroxylation of the β-rings in the carotenoids is

1This appendix is a publication of Quinlan et al., (2012) Plant Physiol. 151: 1635-1645, (www.plantphysiol.org) with permission from the publisher (American Society of Plant Biologists)
potentially mediated by either the P450-type CYP97A or diiron HYD β-ring hydroxylase enzymes. Hydroxylation of the α-ring of α-carotene is performed by another P450 enzyme, CYP97C. Theoretically, a single β-ring hydroxylase should suffice for hydroxylation of the β-ring in both α-carotene and β-carotene. It is unknown why two different β-ring hydroxylases have been maintained throughout evolution; it is possible that their respective activities are not entirely interchangeable. We hypothesized that hydroxylation of each of the carotene rings does not happen independently but that a synergistic interaction occurs between partner enzymes (CYP97A and CYP97C) to facilitate the carotene hydroxylation of α-carotene. To provide support for this hypothesis, we investigated whether carotene hydroxylase enzyme coexpression influenced the biosynthesis of enzyme products. We also determined which enzyme partners showed evidence of physical interaction.

RESULTS

Functional Complementation in Escherichia coli to Test for CYP97 and HYD Substrate Specificity

A widely used functional complementation method employed in our previous studies demonstrated activity of the P450 and HYD carotene ring hydroxylases from grasses (Quinlan et al., 2007; Vallabhaneni et al., 2009). Compared with β-ring hydroxylation by HYD, CYP97A4 and CYP97C2 were less effective in hydroxylation carotene rings in E. coli accumulating β-carotene or α-carotene, their respective β-ring or α-ring substrates. We considered two reasons for the low activity of the CYPs. The first possibility was that the enzymes were not prompted with their optimal substrate, α-carotene, which contains both β- and α-rings. The second possibility was that perhaps the CYP97 enzymes did not function optimally as individual enzymes but required coexpression and interaction, which would allow for efficient hydroxylation of a mixed-ring compound, such as α-carotene. Biochemical phenotypes of plant knockouts support the hypothesis that CYP97 enzymes act sequentially (first CYP97A and then CYP97C) to hydroxylate α-carotene (Kim and DellaPenna, 2006).

We first tested the effectiveness of α-carotene as a substrate, which can only be produced by engineering bacteria to synthesize both α-carotene and β-carotene. We expressed rice (Oryza sativa) CYP97A4 and CYP97C2 (Quinlan et al., 2007) and maize (Zea mays) HYD4 (Vallabhaneni et al., 2009) in E. coli that accumulated both α-carotene (β-α-rings) and β-carotene (β-β-rings). Carotenoid products were analyzed by HPLC and/or liquid chromatography-mass spectrometry (LC-MS). In cells accumulating both α- and β-carotene, the expectation was that hydroxylation of both β-rings in α-carotene by the β-ring hydroxylases (CYP97A and HYD) would lead to formation of the mono-hydroxylated intermediate, β-cryptoxanthin, as well as the end product, zeaxanthin. This was the case for HYD4; cells expressing this enzyme accumulated approximately 80% zeaxanthin. By contrast, cells expressing CYP97A4 mainly accumulated the mono-hydroxylated intermediate β-cryptoxanthin (17% total carotenoids), while only 3% zeaxanthin was generated (Fig. 2; Table I). Similar results were observed when...
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Figure 2. Functional complementation tests of individually expressed CYP97 and HYD enzymes in cells accumulating α- and β-carotene. E. coli cells accumulating both α-carotene and β-carotene were transformed with test plasmids encoding HYD3, CYP97A4, or CYP97C2. Induced products were separated by reverse-phase HPLC, with spectra extracted at 450 nm. Control: Empty pCOLADuet; Z, zeaxanthin, Zε, zeinoxanthin, αε, α-cryptoxanthin; βε, β-cryptoxanthin; εε, 13-cis-β-carotene; αε, α-carotene; βε, β-carotene.

cells were engineered to accumulate β-carotene only (Table II). It was also expected that these β-ring hydroxylases would hydroxylate α-carotene to form zeinoxanthin, and indeed, this product was detected in cells expressing either CYP97A4 or HYD3, although the HYD3 enzyme was twice as active as CYP97A4. In addition, we expected that cells transformed with the α-ring hydroxylase CYP97C2 would accumulate the monohydroxylated product α-cryptoxanthin. However, this compound was barely detected (approximately 0.7% total carotenoids). These results show that HYD3 was most effective in producing a dihydroxylated carotene, in this case zeaxanthin, which was produced from β-carotene. The above results only partially confirmed the hypothesis that P450 carotene hydroxylases (CYP97A and CYP97C) prefer α-carotene over β-carotene as a substrate. CYP97A appeared to function as a monohydroxylase for either β-carotene or α-carotene, but CYP97C was marginally functional, regardless of the substrate. These experiments also showed that CYP97C could not efficiently hydroxylate carotene β-rings. Such a finding was inconsistent with the proposal that CYP97C could hydroxylate both rings of α-carotene to explain the formation of lutein in mutants lacking other known β-ring hydroxylases (Kim et al., 2009). Therefore, we next tested our second

| Table 1. Major products from α- and β-carotene accumulating E. coli with individually expressed hydroxylases. Values are expressed as a percentage of total carotenoids. Each value is the mean of three replicates ± SE. ND, not detectable. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Hydroxylase | Zeaxanthin | α-Cryptoxanthin | Zeinoxanthin | β-Cryptoxanthin |
| CYP97A4 | 3.30 ± 0.27 | ND | 13.63 ± 2.97 | 16.76 ± 2.14 |
| CYP97C2 | ND | 0.71 ± 0.31 | ND | 1.14 ± 0.30 |
| HYD3 | 30.74 ± 1.85 | ND | 23.03 ± 2.72 | 24.03 ± 0.36 |
| Empty vector control | ND | ND | ND | ND |
Table II. Major products in β-carotene-accumulating E. coli with individually expressed hydroxylases

<table>
<thead>
<tr>
<th>Hydroxylase</th>
<th>Zeaxanthin</th>
<th>β-Cryptoxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP97A</td>
<td>11.0 ± 1.1</td>
<td>12.3 ± 0.3</td>
</tr>
<tr>
<td>CYP97C</td>
<td>ND</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>HYD4</td>
<td>29.3 ± 3.8</td>
<td>24.3 ± 1.92</td>
</tr>
<tr>
<td>Empty vector</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

hypothetical, that CYP97A and CYP97C must be coexpressed and physically interact to convert α-carotene to lutein.

When P450 hydroxylases were coexpressed in the presence of α-carotene and β-carotene, their combined activity was dramatically increased, as evidenced by the formation of lutein (25% of total carotenoids), representing hydroxylation of the ε-ring in α-carotene by CYP97C and the β-ring by CYP97A (Fig. 3; Table III). This level of dihydroxyxylated product was comparable to that found for zeaxanthin formation by HYD4 (Fig. 2; Table I). In contrast, coexpression of the β-ring hydroxylase HYD4 with the ε-ring hydroxylase CYP97C2 did not lead to significant levels of hydroxylated carotenoids. Perhaps there was a synergistic interaction occurring between P450 enzymes that did not occur between HYD4 and CYP97C2, since creating a monohydroxylated substrate by HYD4 was insufficient for CYP97C2 to efficiently hydroxylate the remaining ε-ring. Our results showed that the CYP97 enzymes must be coexpressed in order for α-carotene to be fully hydroxylated to form lutein and that the nonheme dirion β-ring hydroxylase (HYD) was not functionally equivalent to the P450 β-ring hydroxylase CYP97A.

The requirement for coexpression suggested that the CYP97 enzymes might interact with each other to produce the dihydroxyxylated carotenoids. We predicted that the interacting enzymes should have similar patterns of plastid localization. Moreover, we expected to detect physical interactions in planta between CYP97A and CYP97C but not between CYP97C and HYD enzymes. To test these predictions, we carried out the following localization experiments.

Plastid Localization of Carotene Hydroxylases Based on Chloroplast Import Studies

Recent proteomic methods utilizing liquid chromatography-tandem mass spectrometry showed CYP97A and CYP97C localized to the Arabidopsis (Arabidopsis thaliana) chloroplast envelope (Joyard et al., 2009; Ferro et al., 2010). However, no data were available for the location of HYD enzymes. Using the online prediction server TMHMM (Krogh et al., 2001), HYD4 was predicted to have four transmembrane helices, which would be expected to confer an integral membrane localization. The CYP97 structures were not predicted to have transmembrane helices. To test whether the hydroxylases were integrally or peripherally associated with membranes, we conducted in vitro chloroplast
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Table III. Major products in α- and β-carotenone accumulating E. coli with compressed hydroxylases

<table>
<thead>
<tr>
<th>Hydroxylase</th>
<th>Lutein</th>
<th>Zeaxanthin</th>
<th>α-Carotenone</th>
<th>Zeinoxanthin</th>
<th>13-Cycloartanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP97A4 + CYP97C2</td>
<td>28.9 ± 2.90</td>
<td>2.98 ± 0.44</td>
<td>ND</td>
<td>ND</td>
<td>7.86 ± 1.28</td>
</tr>
<tr>
<td>HYD4 + CYP97C2</td>
<td>1.58 ± 0.14</td>
<td>3.16 ± 0.13</td>
<td>ND</td>
<td>ND</td>
<td>3.49 ± 0.47</td>
</tr>
<tr>
<td>Empty vector control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are expressed as a percentage of total carotenoids. Each value is the mean of three replicates ± s.d. ND, Not detectable.

import assays (Fig. 4). Radioactively labeled protein precursors were imported into isolated chloroplasts, and then chloroplasts were fractionated into membrane and soluble fractions. CYP97A4 and CYP97C2 proteins were found in the membrane fraction and dissociated from it upon alkaline treatment, indicating that these proteins were imported peripherally. In addition, a significant amount of the CYP97A4 protein was found in the soluble fraction, which also suggested that the peripheral association of the protein is quite weak, allowing the protein to dissociate into a soluble fraction during the fractionation procedure. In contrast, HYD4, found in the membrane fraction as well, proved to be an integral protein, as evidenced by its resistance to alkaline treatment. These data are consistent with our structural predictions and earlier studies of a citrus HYD (Ito et al., 2006).

The functional complementation experiments in E. coli suggested that a synergistic interaction between CYP97A and CYP97C facilitates lutein formation from α-carotenone. Enzyme interactions were tested in plants using the approach of bimolecular fluorescence complementation (BiFC; Gittosky et al., 2006) by transient expression in isolated maize protoplasts. Protoplasts maintain their tissue specificity and reflect in vivo conditions (Farago et al., 2011; Demski et al., 2012) and therefore are valuable for examining enzyme interactions. In addition, transient expression is an advantageous approach for monitoring the localization of low-abundance carotenoid biosynthetic enzymes that evade detection in proteomic studies. In BiFC, putative interacting proteins are fused to nonfluorescent N-terminal and C-terminal halves of the yellow fluorescent protein (YFP); interacting proteins bring together the nonfluorescent fragments, thereby restoring the yellow fluorescence. Constructs encoding fusion proteins were created for CYP97A4, CYP97C2, or HYD4, such that each was fused at their C terminus to N- or C-terminal halves of YFP. The resulting constructs were transiently coexpressed in maize protoplasts and examined using confocal microscopy (Fig. 5A). CYP97A4 and CYP97C2 were found to interact with each other, as shown by restored YFP fluorescence.

![Figure 4. Chloroplast import assays of CYP97 and lutein HYD proteins. Isolated pea chloroplasts were used for in vitro import of [35S]methionine-labeled protein precursors. Chloroplasts harboring imported proteins were then resuspended and subjected to thermolysin treatment to distinguish between proteins that were peripherally bound to the outer chloroplast envelope and those that had been imported and thus processed to remove the transit peptide. The mature proteins were recovered as protease-resistant forms (arrow), confirming the import of these proteins into chloroplasts. Chloroplasts containing imported proteins were hypotonically lysed and fractionated into soluble and membrane fractions. The pellet fractions were then treated with an alkaline buffer to wash away peripherally associated membrane proteins. The purity of fractions was controlled by import and fractionation analysis of a chloroplast luminal protein, tpsOE16::GFP (Marques et al., 2003), and the integral thylakoid membrane-bound protein, LHCP (Tan et al., 2001). SDS-PAGE analysis of chloroplasts and their fractions indicated that CYP97A4 and CYP97C2 were synthesized as precursors of about 69 and 62 KD and then processed to 64 and 59 KD, respectively. HYD4 was synthesized as a precursor of roughly 14 KD and processed to 37 KD. P, Translation product; I, imported protein; +, thermolysin treatment; S, soluble protein; M, membrane proteins; MA, alkaline-treated membrane fraction.](image-url)
interaction of CYP97A4 and CYP97C2 was additionally confirmed by an in vitro pull-down assay (Fig. 6). We also detected a HYD4 + HYD4 interaction, which suggested that HYD4 formed a homodimer. We did not detect homodimers for CYP97A4 or CYP97C2 or heterodimers for CYP97A4 + HYD4 or CYP97C2 + HYD4 (Supplemental Fig. S2). Interaction results for all tested protein combinations are summarized in Table IV. For comparison, we also individually expressed the enzymes as GFP fusions to confirm plastid localization in our protoplast system (Fig. 5B). A similar fluorescence pattern indicates that the interaction does not change protein localization as seen for the individually expressed proteins.

**SYNGNOSTIC ENZYMES IN CAROTENOSIS**

**Figure 6.** Pull-down assay. Interaction of CYP97A4 and CYP97C2 was shown in vitro by pull-down assay. CYP97C2 was expressed and purified from E. coli cells carrying pGEX-CYP97C2 in the column and used as bait for CYP974A. Radioactively labeled CYP97A4 interacted with CYP97C2 and interacting proteins eluted from the column together. Control loading of CYP97A4 to pure NTA did not show any nonspecific binding. Top, autoradiograph of a SDS-PAGE gel, showing CYP97A4 from the in vitro translation reaction and CYP97A4 in the eluate from the NTA + CYP97C2 column; bottom, Coomassie blue staining of the same gel.

**DISCUSSION**

**Interacting Proteins Exhibit Synergistic Effects on Carotene Dihydroxylation**

Using a bacterial assay system, we showed that dihydroxylation of α-carotene to lutein requires the coexpression of CYP97A and CYP97C, enzymes that interact in planta and interact in vitro in pull-down assays. In contrast, lutein does not form in the case of enzymes that do not exhibit interaction, such as HYD and CYP97C. We hypothesize that a synergistic interaction between CYP97A and CYP97C is required to drive lutein biosynthesis by the purpose of channelling pathway substrates, stabilizing the enzyme-substrate complex and/or promoting interaction with other enzymes or components. We also found that the most efficient dihydroxylation of β-carotene to zeaxanthin was achieved by HYD, an enzyme that could form homodimers. CYP97A, which could not form homodimers, was less efficient in the dihydroxylation of β-carotene to zeaxanthin. Although further research is needed to understand the connection between interaction and the efficiency of dihydroxylation in planta, we hypothesize that the ability to form a protein complex improves the efficiency of hydroxylation of dual-ringed carotene substrates.

**Do CYP97A and CYP97C Work Simultaneously or Sequentially?**

The hypothesis based on plant mutants is that in carotenoid biosynthesis, CYP97A functions first to produce the monohydroxylated carotene, zeaxanthin, which is transferred by some unknown mechanism as
the substrate for CYP97C (Kim and DellaPenna, 2006; Kim et al., 2009). In these studies, CYP97C mutants accumulated substantially higher levels of the monohydroxylated carotene, compared with CYP97A mutants, although the levels were not what would be expected on the basis of wild-type levels of lutein formed in leaf tissue. In CYP97A mutants, the monohydroxylated α-cryptoxanthin barely accumulated, indicating that CYP97C was unable to hydroxylate the α-ring of α-carotene. Using the bacterial assay system, we found that CYP97A produced approximately 20-fold more monohydroxylated carotene (zeaxanthin) as compared with levels of monohydroxylated carotene (α-cryptoxanthin) catalyzed by CYP97C, when either enzyme was expressed alone in bacterial cells producing α- and β-ring-containing carotenes. These results show that in bacteria, CYP97A can accept the α-carotene substrate to produce the monohydroxylated product, while CYP97C is limited. Of course, there are many reasons why CYP97C might show poor activity in E. coli (e.g., nonoptimized codon usage, missing cofactors). However, the fact that CYP97C can function in bacteria when coexpressed with CYP97A suggests that CYP97A is the missing factor that must be present in order for CYP97C to function and, together with CYP97A, to produce lutein. The observed interaction between these two enzymes may reflect a stabilizing complex required in the case of CYP97A activity and a multienzyme structure needed to channel the zeaxanthin substrate in the case of CYP97C. We propose that protein-protein interaction between CYP97A and CYP97C facilitates the recruitment of CYP97C2 to access the zeaxanthin substrate. Such interaction might also serve in a regulatory role to control pathway flux. It would be intriguing to learn whether these enzymes exist only as a heterodimer or if other proteins, including other carotenoid enzymes, also participate in the formation of a metabolon in vivo.

### Substrate Specificity?

From genetic and functional complementation studies (Tian et al., 2004; Kim and DellaPenna, 2006; Quinlan et al., 2007), it is generally accepted that CYP97C is an α-ring hydrolase and CYP97A is a β-ring hydrolase. Mutant phenotypes suggested that CYP97A hydroxylates the β-rings of α-carotene to form zeaxanthin, which is the preferred substrate for α-ring hydroxylation by CYP97C (Kim and DellaPenna, 2006). However, biochemical phenotypes of mutants carrying only a subset of carotene ring hydroxylases suggest broader substrate specificity for these enzymes. Arabidopsis mutant plants with only CYP97C (and not CYP97A) or the two nonhemic carotene hydroxylases produced significant levels of lutein, approximately 75% of the wild type, and plants with only CYP97A contained lutein at about 5% of the wild type level (Kim and DellaPenna, 2006; Kim et al., 2009). The explanation given for the significant level of lutein, which requires both ring-specific hydroxylases although only one is present, is that the remaining CYP has additional activity toward the other ring type. CYP97C, in particular, was thought to have significant β-ring hydroxylation activity, given that the triple mutants still produced 25% of wild-type levels of lutein (Kim et al., 2009). Broad substrate specificity for CYPs was also suggested by the results of CYP97A overexpression (Kim et al., 2010). However, in our earlier E. coli functional complementation studies (Quinlan et al., 2007), we found no evidence that the α-ring hydroxylase CYP97C could hydroxylate β-rings, and in this study, β-ring hydroxylation by CYP97C was minimal. One explanation (Kim et al., 2009) for the disparity between the apparent function of CYP97C in plants and in E. coli was that the engineered E. coli contained only the individual β-ring or α-ring substrates but not the mixed-ring substrates found in plants. Therefore, if CYP97C did indeed hydroxylate both β- and α-rings, as postulated, we should have obtained lutein accumulation in bacteria that produce both β- and α-rings as evidence of such postulated broad substrate specificity. However, even when simultaneously presented with both the β- and α-rings, neither CYP97C nor CYP97A, when expressed alone, produced detectable levels of lutein and in general barely produced a dihydroxylated product. Therefore, we found no evidence for broad substrate specificity to explain lutein formation by a single CYP enzyme, as suggested by the plant studies. The question remains, why is lutein still formed in mutants containing only CYP97C? Recent studies (Kim et al., 2010) suggest that another poorly studied paralog, CYP97B, which is evolutionarily related to CYP97A and CYP97C, might exhibit a carotene β-ring hydroxylation activity. If so, then an explanation for the production of lutein in the Arabidopsis triple mutants is the functional CYP97B that is present in the mutant genetic background. If CYP97B is indeed a β-ring hydroxylase, it might function together with CYP97C to form lutein. Similarly, if CYP97B has some minor α-ring hydroxylation activity, CYP97B could form lutein in partnership with CYP97A. In fact, in triple Arabidopsis mutants, levels of lutein were lower when the "only" ring hydroxylase was CYP97A as compared with when the "only" ring hydroxylase was CYP97C. Therefore, one could speculate that CYP97B is more efficient in hydroxylating β-rings as compared with α-rings. Furthermore, we might expect CYP97B to form functional enzyme partnerships, as we observed for CYP97A and CYP97C. That is, expression of CYP97B alone would be predicted to be insufficient to mediate carotenoid dihydroxylation (e.g., to produce xanthophylls...
such as lutein). In support of this hypothesis, quadruple mutants (CYP97A, CYP97C, and the two nonhomocarotene hydroxylases) of Arabidopsis were allayed and showed 10% of wild-type levels of carotenoids, but xanthophylls were completely blocked (Kim et al., 2009). Absence of a requisite partner enzyme could explain why xanthophylls are not produced in the quadruple mutant even in the presence of CYP97B. Further enzyme analysis of CYP97B and studies to test for synergistic interaction with the other carotene hydroxylases are warranted.

Enzyme Interchangeability and Pathway Dead Ends

Since both HYD and CYP97A have the ability to produce zeaxanthin by hydroxylation of the β-ring of α-carotene, CYP97A and HYD were expected to be functionally interchangeable. However, the results of enzyme coexpression did not support this. Combined expression of HYD and CYP97C was not productive in lutein formation in bacteria, showing that HYD could not substitute for CYP97A, which is consistent with a similar conclusion based on the biochemical phenotypes of plant mutants (Kim and DellaPenna, 2006). If interaction between CYP97C and CYP97A is needed to stabilize carotene ring hydroxylations at two nonenzymes (e.g., by providing zeaxanthin substrate), then the inability to form a complex could account for the inability of HYD and CYP97C to function together. It is possible that a noninteraction has nonfunctional explanations. However, the absence of interaction was consistent with the lack of synergy seen in bacterial coexpression and may be further explained by the fact that the enzymes exist in different membrane settings. Therefore, if and when HYD catalyzes the formation of zeaxanthin from α-carotene in plants, this zeaxanthin will be a pathway dead end in terms of further conversion to lutein.

CONCLUSION

Based on our results, we conclude that in the branched pathway in plants, the primary route to the formation of lutein from α-carotene is mediated by HYD enzymes, whereas the primary route for zeaxanthin formation from β-carotene is mediated by CYP97A enzymes with some contribution from CYP977.

Our studies also support the widely held notion that carotenoid biosynthesis involves protein complexes to maximize pathway flux. Such an interaction between proteins is a useful regulatory mechanism that allows plants to direct the pathway toward various metabolites, as required in certain tissues or conditions. Our studies showing an interaction of these proteins, to our knowledge for the first time, lay the foundation for further investigations into the roles and topologies of the putative carotenoid metabolites. Further understanding of protein-protein interactions in the pathway will provide insight for more efficient engineering of carotenoid conversion to avoid dead-end products and improve plant stress responses or nutritional content.

MATERIALS AND METHODS

Cloning of CYP97A, CYP97C, and HYD4

Amplification of open reading frames for cloning was performed by Platinum PCR SuperMix High Fidelity Starter Mix (Invitrogen) according to the manufacturer’s instructions. PCR conditions were as follows: one cycle of 96°C for 1 min; 35 to 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 to 2.5 min; and one cycle of 72°C for 10 min. For primers, see Supplemental Table S1.

cPOLADuct and pCDFDuct Constructs Used for Expression in Escherichia coli

For cloning into pCOLADuct vector (Novagen), full copies of complementary DNA (cDNA) of CYP97A4 and CYP97C2 were amplified from rice (Oryza sativa) cDNA (Quandt et al., 2007). pCOLADuct-CYP97A4 was renamed pT7-4A. CYP97C2 was amplified from pCLOT7-2-CYP97C2 using primers 2250 and 2191, cloned into XbaI and AatII sites of pCDFDuet-1 vector (Novagen), and renamed pT7-2C. HYD4 was amplified from pfHYD4 (Vitalovskaya et al., 2008) using primers 1832 and 1834 and cloned into pCLOT7-2-C. pCOLADuct-HYD4 was renamed pT7-4D.

pTnAT Constructs Used for in Vitro Transcription/Translation

A full-length cDNA of CYP97A4 was amplified from the pTnAT vector via PCR with primers 2175 and 2177. CYP97A4 was amplified from rice cDNA using primers 2177 and 2181. HYD3 was amplified from pTnAT4 using primers 2183 and 2187. CYP97A4 and CYC97C2, and HYD4 were cloned into the XbaI and SalI sites of the pTnAT vector (Novagen) and named pT7-4A, pT7-2C, and pT7-4D, respectively.

pUC555-GUS-Nos Constructs Used for in Plant Localization

A full-length cDNA of CYP97A4 without the stop codon of the CYP97A4 open reading frame was amplified from the pT7-4A vector with primers 2184 and 2205. CYP97A4 was amplified from pGEMT-C2 (pGEMT-Easy vector [Promega] harboring a full-length cDNA of CYP97C2 using primers 2579 and 2200. HYD4 was amplified from the pGEM- H4 using primers 2620 and 2631. CYP97A4, CYC97C2, and HYD4 were cloned in frame with GUS into the XbaI and SalI sites of the pUC555-GUS-Nos vector (based on pUC555-GUS-Nos and pCLE21 vector; Okuda et al., 2003) and named A1-GFP, A2-GFP, and H1-GFP, respectively.

pSAT Constructs Used for BtC Experiments

For cloning into pSAT-2226 (pSAT7(A3-pEYP-N3)), a full-length cDNA without the stop codon of CYP97A4 was amplified from pT7AT4 using primers 2425 and 2424 (Clowery et al., 2008). CYP97C2 was amplified from pGEMT-C2 using primers 2425 and 2424. HYD4 was amplified from pT7AT4 using primers 2425 and 2424. CYP97A4, CYC97C2, and HYD4 were cloned into the XbaI and SalI sites of pSAT-2226, and named A1, A2, and H1, respectively. CYP97C2 was cloned into NcoI and EcoRI sites of pSAT-2226 and named A2, A1, and H1, respectively. CYP97C2 was cloned into NotI and SalI sites of pSAT-2226 and named A1, A2, and H1, respectively.
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pET23b+ Construct Used for Expression and Purification

The pET23b+ plasmid was amplified from pET23c+ with primers 3230 and 3210 and cloned into pET23b (Merck) to give a translational fusion with a His tag. The resulting plasmid was named pET23c+TPC1. 

Functional Analysis of Hydroxylases in E. coli

The expression of the enzymatic activity for individual enzymes, pET-glut, pET-PC2C, or pET-H14 was confirmed to be 100% of the total activity encoded by the adjacent promoter. The activity of each enzyme was determined as follows: (1) pET-ET-A1 (Cunningham et al., 2007) only contained anarthesis activity, (2) pET-A1 (Cunningham et al., 2007) only contained anarthesis activity, (3) pET-H14 (Munro et al., 2007) only contained anarthesis activity, and (4) pET-ET-ET-A1 (Cunningham et al., 2007) only contained anarthesis activity. For testing of specific activity of enzyme combinations, the pET-ET-A1 + pET-ET-ET-A1 constructs were transformed into E. coli BL21 (DE3) cells (Novagen) harboring both pET-ET-A1 and pET-ET-ET-A1, and further cultured for a total of 30 mL. Negative controls, i.e., bacteria-purifying controls, were transformed with empty vectors. 

Extraction of Carotenoids from E. coli cells, HPLC, and LC-MS Analysis

Fifty-milliliter cultures containing antibiotics for selection of plasmids (ampicillin, 50 µg mL⁻¹; chloramphenicol, 34 µg mL⁻¹; kanamycin, 30 µg mL⁻¹; streptomycin, 30 µg mL⁻¹) were centrifuged at 3000 × g for 10 min. Supernatants were decanted and the cells resuspended in 5 mL of methanol using a vortex (Vita; SBS) and pelleted down at 3000 × g for 10 min. Supernatants were transferred to 10 mL Pyrex flasks and evaporated under nitrogen gas, then dissolved in 300 µL of methanol and 300 µL of methanol and 300 µL of methanol. Supernatants were transferred to HPLC vials (Waters). 

HPLC separation was carried out using a Waters system equipped with a 250 × 4.6 mm, 5 µm, C18, 200-A polar column at 25°C. A gradient of 0.1% formic acid in water (Mobile A) and 0.1% formic acid in acetonitrile (Mobile B) was used for separation. The flow rate was 1 mL min⁻¹, and the UV-Vis detector was set at 450 nm. All data were collected from 450 mL. 

Chlorophyll Isolation and in Vitro Import

Chlorophylls used in import assays were isolated from 60 to 100-µmolPhoton (antron) plants as described (Birks et al., 1949). Approximately 25 g of leaves was homogenized at 4°C with a blender in 30 mL of cold grinding buffer (0.1 mollH₄PO₄, pH 6.0, 0.3% sodium, 1 mol NaCl, 1 mol NaCl, 2 mol NaEDTA, pH 8.0, 0.1% bovine serum albumin, and 0.1% NaN₃) by three bursts of 1 sec each. All further operations were performed on ice using cold buffers. The homogenate was filtered through two layers of cheesecloth and the layer of nylon mesh (60 µm) and the filtrate was centrifuged at 2000 × g for 2 min. The supernatants were carefully resuspended in 1 mL of grinding buffer, sonicated on top of 1 mL of cold grinding buffer (0.1 mollH₄PO₄, pH 6.0, 0.3% sodium, 1 mol NaCl, 1 mol NaCl, 2 mol NaEDTA, pH 8.0, 0.1% bovine serum albumin, and 0.1% NaN₃) by three bursts of 1 sec each. All further operations were performed on ice using cold buffers. 

Isolation and Transformation of Maize (zea mays) Protoplasts

Isolation and transformation of maize (zea mays) protoplasts was performed using protocols from Stern (1991) and van Beekveldt et al. (1990), as a guide, with modifications. Nuclear var 17D plants were grown in the dark at 2°C for 24 h (12 h of day, 12 h of night) and protoplasts were cut into razor-thin sections and transferred to 50 mL of calcium/mannitol solution (10 mM CaCl₂, 66 mM mannitol, and 20 mM MES, pH 5.7) to which was added 1% dextrose (1% dextrose, 0.5% Cellulose microparticles, Sigma), 5% agarose (Sigma), and 0.5% bovine serum albumin (Sigma). Vacuole acidification was achieved by shaking at 60 rpm in a water bath to 2°C in a dark. The suspension was filtered by 0.45 µm polycarbonate (Millipore) and filtered with 0.45 µm polycarbonate (Millipore). Protoplasts were pelleted at 600 × g for 5 min at room temperature. The supernatant was discarded and the protoplasts were resuspended in 1 mL of calcium/mannitol solution and pelleted at 600 × g for 5 min at room temperature. The suspension was resuspended in 1 mL of calcium/mannitol solution and pelleted at 600 × g for 5 min at room temperature. 

Confocal Microscopy

Confocal microscopy of GFP or YFP fusion proteins was visualized using a BX51 microscope equipped with a 60X, 1.40 NA oil immersion objective and a 1.25× Salfocam. Confocal microscopy of expression was carried out in a 100-mm Petri dish. The nucleus was then observed using the same instruments with a 1.40 NA oil immersion objective and a 1.25× Salfocam. Confocal microscopy of expression was carried out in a 100-mm Petri dish.
of the organ laser was used to excite the fluorescence of YFP, and the emission spectrum was detected and confirmed by a scan between 310 and 570 nm. All software (Luna Imaging System) was used for image acquisition. Images were obtained by combining several confocal Z-planes.

**In Vitro Pull-Down Assay**

pEII2-CYP79C2 encoding a fusion protein was expressed in the C3H (CE2) strain of E. coli (Lucigen). Cells were grown in 250 ml yeast extract and tryptone (2x VT) medium containing 50 mg ml-1 ampicillin until an optical density of 6.0, and protein expression was induced by adding 1 ml isopropyl-β-D-thiogalactoside, followed by incubation at 30°C overnight. Pelleted cells were resuspended in 25 ml of lysis buffer [0.1 M HEPES, pH 7.6, 300 mM NaCl, 20 mM imidazole, 2 M glycerol, 0.1% (w/v) NaDC (Nonidet), 0.02% 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 10% glycerol]. Lysed cells were then sonicated and diluted with 40 ml of lysis buffer containing 1% Triton X-100 and 10% glycerol. Samples were used at 4°C for 1 h. Non-detachable material was removed by centrifugation at 10,000 rpm for 30 min at 4°C.

Fusion proteins were double-passed through a 0.3 μm syringe filter membrane and concentrated to ~20 ml. Samples were then loaded onto a 1 ml Ni-NTA column and eluted overnight at 4°C. Two hundred microliters of the eluted sample containing recombinant CYP79C2 was used for the pull-down assay.

Fifteen minutes of 5%-loaded CYP79A4 protein translocated in vitro from pE2A-A4 was added to 400 μl of diluting buffer (25 mM HEPES, pH 7.6, 150 mM NaCl, and 40 mM imidazole). CYP79A4 solution was mixed with the CYP79A2 assay, and incubated for 2 h at 4°C. The same amount of CYP79A2 was mixed with 300 μl of pure NADPH as a negative control. After incubation, samples were loaded onto 10% polyacrylamide gels (4:1) and electrophoresed with 4% polyacrylamide gels, and then stained with 0.1% Coomassie Blue. The gel was stained with CBB R-250 for total protein and dried before photogating.

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers for rice: CYP79A4 (AA320650) and CYP79C2 (AA320650); for maize, H74G (BX289707/5/1/8/8556).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Extracted ion chromatogram of mass spectrometry traces corresponding to the major quinone-arylation and formylated CYP79A4 ion generated by tandem mass spectrometry.

**Supplemental Figure S2.** HCR assays demonstrating no protein interaction for the indicated CYP79C2/VIS1 combination tested using proteo iplotted isolated native leaf tissue.

**Supplemental Table S1.** Genes used in the study.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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## Appendix 6: Plasmids used in this study and laboratory clone sheets

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<td>pACCRT-EBP+pCola pCOLADuet-1 Empty BI21 (DE3) competent cells harboring pACCRT-EBP (#150) were transformed with pCOLADuet-1 empty (Chapter 2)</td>
<td></td>
</tr>
</tbody>
</table>

**Appendix table 6.1.** List of plasmids used in this study and laboratory clone sheets.
## Clone Description:

ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 135 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*.

### Primers used for amplification from ZmZ-ISO1

<table>
<thead>
<tr>
<th>Fragment size</th>
<th>Number</th>
<th>5’ Sequence</th>
<th>3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG GGATCC TATGGCCTCCCAGCTCCGCCTC 1,108</td>
<td></td>
</tr>
<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGAGCT</td>
<td></td>
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</tbody>
</table>

**Constructed by:** Jesús Beltrán, 2010  
**Purified by:** Jesús Beltrán  
**DNA Location (-20°C):** Box Number # 10 Position: D3 Conc. 20ng/ul  
<table>
<thead>
<tr>
<th>Tube labeled as:</th>
<th>pCol Zm-ZISO 135 H/A</th>
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</thead>
<tbody>
<tr>
<td><strong>Strain Location (-80°C):</strong></td>
<td></td>
</tr>
<tr>
<td>XL10 Gold:</td>
<td>Box Number: 17 Position: F4. tube labeled as: pCol Zm-ZISO 135 H/A</td>
</tr>
<tr>
<td>EBP:</td>
<td>Box Number: 17 Position: G7. tube labeled as: EBP pCol Zm-ZISO 135 H/A</td>
</tr>
</tbody>
</table>

**NOTE:** This mutation does not affects Zm ZISO activity  
Sequence verified: Yes  Junction verified: Yes

TCACTATAGGGGAAATTGTGAGCCGATAAACCAATCCCTGTAAGAAATAATTTTTGTTT
AACTTTAATAAGGAGATATACCATGGGCAGCAGCCATACACCCTACACCACCACAGC
CAGGATCATCTA4TGCCCTCCCGCTCCGCTCCACCTTTCGCGCTACACCAGCCTCTCTC
TCCTCAACCGCCGTCGCCACCTCCGCGCCGCACGCTCTGCCACCCCTAACACCACATCC
GAGCGCCGCTCCCCCCTCTCTACCGGTTCCTCTCCCCAACGCTCGTCCGGCCGCCGCTG
GCTCGGCGGAGCCTTGAGCCGCAAATGAGAGCTTCTGTTGTGCTTTCGCGCTACCCAC
CATCACCATCACCAC
AGC
CAGG
ATCCT
ATG
GCCTCCCAGCTCCGCCTCCACCTTGCGGCTACACCGCCTCTCCT
TCCTCACCGCCGTCCGACCTCCCGAGCGCCGCTCCCCCCTCTCTCACGCGTTCTCTCCCACGCTCGTCCCGCCGCTG
GTCGGGGGAGGCATCGAGCCGAAGGAGGGCGTTGTTGGAGGGAGACGAGTCT
GGCGGAGGGCCCCTGCTCGTGGGTTGAGGATTCGCGCTCGTCGTCCAGCTCAGAGACC
AGAGCGTGCCGCTGCTGGCTTAATCTGCCTGCCGGGATACATGTGCGTGCTGCTCTGC
GCTCAACGTGCTGTGGAATCGACCCCAGTAGCAAGGGGGTCGGGACCCAAATTCCTCGAC
GCTGTCGCCCCTCCGCTCCTCCAGCGCCAGCGAGTTGTTATTGTTGCTCCTTACCATAAT
TTTTGCTGTAGTCCATAGTTGGATGGCAAGCCACTACGGGAAGGTGGTGAGAAAAATA
GTTGGGGGACGGTTTACCCTGCGTTGCTGTTGCTGGAATTTTACCTCGCTCTTAGCAGT
TACTACTATTTGATACTTCAATAATCATCGGTATGATGTTACTCAATTATGCAAG
TTCAAGGGAATCAGTGCCATTACATGACGCTCTTTGTTTCTCGTCTCTGTTATCTCTCT
CTCTTTGTATCCATCCATCCTCTTTGTTTCTCTGCTGTTATCTCTTTGTAAGGAGCT
TGTAGCAATCACAATGTTAACCTTGGGT
GCATACTTTGCTCATCCATTGATGCAAGCATCCAGCTACCACTTCCCTGG
TAG
GT
CGACAAG
NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
**WURTZEL LAB**

**CLONE INFORMATION**

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<td>Clone type; Expression cDNA clone</td>
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<table>
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<tr>
<th><strong>Clone Description:</strong></th>
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<tbody>
<tr>
<td>ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H150 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: <em>BamHI</em> and <em>SalI</em></td>
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</table>

<table>
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<th>Primers used for amplification from ZmZ-ISO1 are</th>
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<td>fragment size</td>
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<tr>
<td>ZmZ-ISO-BamHI</td>
</tr>
<tr>
<td>1,108</td>
</tr>
<tr>
<td>ZmZ-ISO-SalI</td>
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| Constructed by: Jesús Beltrán, 2010 |
| Purified by: Jesús Beltrán |
| DNA Location (-20°C) Box Number # 10 Position: D5. Conc. 20ng/ul |
| Tube labeled as: pCol Zm ZISO 150H/A |
| Strain Location (-80°C) |
| XL10 Gold : Box Number: 17 Position: F6. tube labeled as: pCol Zm-ZISO 150 H/A |
| EBP : Box Number: 17 Position: G9. tube labeled as: EBP pCol Zm-ZISO 150 H/A |

| NOTE: This mutation affects Zm ZISO activity |

| Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán |
| Original clone name (if different): |

| Organism source of gene: Maize B73 |
| Cloning vector used: pColaDuet-1, Strain: XL10 Gold |
| Vector size: 3719bp Insert size: 1,108bp |

<p>| Antibiotic markers: Kanamycin |
| Restriction enzyme(s) to release insert: <em>BamHI</em> and <em>SalI</em> |</p>
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<td>AGGATCCTAGTGCCCTCCAGCTCCGCCCTCCACCTTGCGCTACACCAGCGCTCCTTCT</td>
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NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
**WURTZEL LAB**

**CLONE INFORMATION**

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<td>Clone type: Expression cDNA clone</td>
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**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 191 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

Primers used for amplification from ZmZ-ISO are:

<table>
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<tr>
<th>Number</th>
<th>5’ Sequence 3’</th>
</tr>
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<tbody>
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<td>fragment size</td>
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<tr>
<td>ZmZ-ISO-BamHI 2586</td>
<td>CG <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
</tr>
<tr>
<td>1,108</td>
<td></td>
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<tr>
<td>ZmZ-ISO-Sal I 2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGGAAGTTGGTAGCT</td>
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**Constructed by:** Jesús Beltrán, 2010

**Purified by:** Jesús Beltrán

**DNA Location (-20°C):** Box Number # 10 Position: D7 Conc. 20 ng/ul

- Tube labeled as: pCol Zm ZISO191 H/A
- Strain Location (-80°C): XL10 Gold: Box Number: 17 Position: F8. Tube labeled as: pCol Zm-ZISO 191 H/A
- EBP: Box Number: 17 Position: H2. Tube labeled as: EBP pCol Zm-ZISO 191H/A

**NOTE:** This mutation does not affects Zm ZISO activity


**Lab Notebook to reference:** Jesús Beltrán, Depositor’s Name: Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** Maize B73

**Cloning vector used:** pColaDuet-1, Strain: XL10 Gold

**Vector size:** 3719bp **Insert size:** 1,108bp

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:** BamHI and SalI
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<th>Junction verified: yes, in the original clone</th>
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**NOTES:** Conditions for expression are essential as reported by Chen et al., 2010. 
**Underline seq.:** His tag
**WURTZEL LAB**  
**CLONE INFORMATION**

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<td>Clone type:</td>
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**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 266 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

Primers used for amplification from ZmZ-ISO1 are

<table>
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<tr>
<th>fragment size</th>
<th>Number</th>
<th>5’ Sequence 3’</th>
</tr>
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<tbody>
<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>GC <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
</tr>
<tr>
<td>1,108</td>
<td></td>
<td></td>
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<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGAAGTTGGTAGCT</td>
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</tbody>
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**Constructed by:** Jesús Beltrán, 2010  
**Purified by:** Jesús Beltrán  
**DNA Location (-20°C) Box Number # 10 Position: D9 Conc. 20ng/ul**  
Tube labeled as: pCol Zm-ZISO 266 H/A  
**Strain Location (-80°C)**  
XL10 Gold : Box Number: 17 Position: F10. tube labeled as: pCol Zm-ZISO 266 H/A  
EBP : Box Number: 17 Position: H4. tube labeled as: EBP pCol Zm-ZISO 266 H/A

**NOTE:** This mutation affects Zm ZISO activity

**Cited in journal:**  

**Lab Notebook to reference:** Jesús Beltrán, Depositor’s Name: Jesús Beltrán  
**Original clone name (if different):**

**Organism source of gene:** Maize B73  
**Cloning vector used:** pColaDuet-1, Strain: XL10 Gold  
**Vector size:** 3719bp **Insert size:** 1,108bp  
**Antibiotic markers:** Kanamycin
Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Site of mutation verified  Junction verified: yes, in the original clone

ACTCACTATAGGGAAATTGAGCCGATAAACAAATTCCTCTGTAAGAAAAATAATTAGTTTATTAACCTTAAAAAGGAGATACGACCTCAGTCCACCTCACGATCCACGCTCTCCCGGATCGGTCACGCTATGGTGCTGCTCCGACATTAGCAATCAATGTTAACCTTTGGTGCTCCATTCCATTGAG

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
Clone Number/Name: pCol Zm ZISO 285H/A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 285 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<tr>
<th>Fragment size</th>
<th>Number</th>
<th>5’ Sequence 3’</th>
</tr>
</thead>
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<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG GGATCC TATGGCCTCCCAGCTCCGCCTC</td>
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<tr>
<td></td>
<td>1,108</td>
<td>1,108</td>
</tr>
<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC GTGCAG CTACCAGGGAAGTTGGTAGCT</td>
</tr>
</tbody>
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Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 10 Position: E1 Conc. 20ng/ul
 Tube labeled as: pCol Zm-ZISO 285 H/A

Strain Location (-80°C)
XL10 Gold : Box Number: 17 Position: G3. tube labeled as: pCol Zm-ZISO 285 H/A
EBP : Box Number: 17 Position: H6. tube labeled as: EBP pCol Zm-ZISO 285 H/A

NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán

Original clone name (if different):

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1, Strain: XL10 Gold
Vector size: 3719bp Insert size: 1,108bp

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI
<table>
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<tr>
<th>Sequence verified: Site of mutation verified</th>
<th>Junction verified: yes, in the original clone</th>
</tr>
</thead>
</table>
| CACTATAGGGGAATTGTGAGCGGATAACAATTCCCTCTGTAGAAATAATTGTGTTTACTTTAATAAGGAGATATACCATGGGCAGCAGCCAATCACATCATCACACCACAGCC | CACTCTAGGGGCACTCCCGAGCTCCCGGCCTTCCTACCCGCTCCGCCCTACTCCCTCTCCGCTCCGCCCTCCTCCTTTCCTCACCGCCTCCGCCTCCTCCTCGCCCTTCCGGCTCCTCCTTCCTCACCGCCGTCCGCACCTCCCGGCCGCCGCCGCTCTGCCCCACCCTAAAACCCTACATCGCGAGCTCGTGGTGATGGCAGGCCGCTCCCCCCTCTCCTACCTCAGGCTCTGTATAATTTGCTGTAGTCTCGTCGTTGCTCGACATGCTAAAGGCTGAGCAGCAGCAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAAAGGCTGAGGAGTAA4

**NOTES:** Conditions for expression are essential as reported by Chen et al., 2010.

Underline seq.: His tag
WURTZEL LAB
CLONE INFORMATION

Date Today:  7/12/2010
Entered into database yes/  # 526

| Clone Number/Name: pCol Zm ZISO 286H/A |
| Clone type: Expression cDNA clone |

**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 286 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

Primers used for amplification from ZmZ-ISO1 are

<table>
<thead>
<tr>
<th>Fragment size</th>
<th>Number</th>
<th>5’ Sequence 3’</th>
</tr>
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<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
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<tr>
<td></td>
<td>1,108</td>
<td>1,108</td>
</tr>
<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGGAAGTTGGTAGCT</td>
</tr>
</tbody>
</table>

**Constructed by:** Jesús Beltrán, 2010
**Purified by:** Jesús Beltrán

**DNA Location (-20°C) Box Number # 10 Position: E3 Conc. 20ng/ul**
- Tube labeled as: pCol Zm-ZISO 286 H/A

**Strain Location (-80°C)**
- XL10 Gold: Box Number: 17 Position: G5. tube labeled as: pCol Zm-ZISO 286 H/A
- EBP: Box Number: 17 Position: H8. tube labeled as: EBP pCol Zm-ZISO 286 H/A

**NOTE:** This mutation does not affects Zm ZISO activity


**Lab Notebook to reference:** Jesús Beltrán, Depositor’s Name: Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** Maize B73

**Cloning vector used:** pColaDuet-1, Strain: XL10 Gold
**Vector size:** 3719bp **Insert size:** 1,108bp

**Antibiotic markers:** Kanamycin
Restriction enzyme(s) to release insert: BamHI and SalI

<table>
<thead>
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<th>Sequence verified: Site of mutation verified</th>
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<td>TTGCTCATCTATGATGCAAGCATCAGCTACCAACTTCCCTGGTAGGTT</td>
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NOTES: Conditions for expression are essential as reported by Chen et al., 2010.

Underline seq.: His tag
WURTZEL LAB
CLONE INFORMATION

Date Today: 7/12/2010

Entered into database yes/ # 527

| Clone Number/Name: pCol Zm ZISO 47H/A |
| Clone type: Expression cDNA clone |

**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 47 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *Bam*HI and *Sal*I

Primers used for amplification from ZmZ-ISO1 are

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<th>fragment size</th>
<th>Number</th>
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<tr>
<td>ZmZ-ISO-BamHI</td>
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<td>CG <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
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<tr>
<td>1,108</td>
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<td>1,108</td>
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<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGGAAGTTGGTAGCT</td>
</tr>
</tbody>
</table>

Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 10 Position: E9 Conc. 20ng/ul
- Tube labeled as: pCol Zm-ZISO 47 H/A
Strain Location (-80°C)
- XL10 Gold: Box Number: 17 Position: H10. tube labeled as: pCol Zm-ZISO 47H/A
- EBP: Box Number: 17 Position: J4. tube labeled as: EBP pCol Zm-ZISO 47H/A

NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán

Original clone name (if different):

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1, Strain: XL10 Gold
Vector size: 3719bp Insert size: 1,108bp

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: *Bam*HI and *Sal*I
Sequence verified: Site of mutation verified  Junction verified: yes, in the original clone

| TTTAACTTTTAATAGAGGTATATACCATGGGACGAGCCATCCACCACTGACACCACACCACAGCAGGATCCTAGTGCTCCTCCAGTCCGGCTCAACCAGGCCTCTTGGGATCCTTGGCCTTCGATCACCAGGAGTTACAAGGTCGACGAGGACGACAGGAGTTGCTTTTGCCAGGTTTACGCTGTGACGTCGGTGGGCTTATAGGCAGGTGTCATTGCTGCCCATGCTGGTTGTTTGCAGGATCGAATAGGTGAAGCTGAAGAAGAGAACAAGTGTTATGCCCTTCGCTGCGATCATCGATGGACGGCAGAAACTGCCCAAGGATTATCACAAGGAGTTCTTTCGGTTACCATAAGTTAGCAATCACAATGTTAACCTTGGGTGCATACTTTGCTCATCCATTGATGCAAGCATCCAGCTACCACTTCCCTGGTTAGGTCTGA |

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
## CLONE INFORMATION

**Date Today:** 7/12/2010  
**Entered into database:** yes/  
**# 528**

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<tbody>
<tr>
<td>Clone type</td>
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### Clone Description:

ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 208 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

Primers used for amplification from ZmZ-ISO1 are

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<th>fragment size</th>
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<th>5’ Sequence 3’</th>
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<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG <em>GGATCC</em> TATGGCCTCCCAGCTCCGCCTC</td>
</tr>
<tr>
<td>1,108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC <em>GTCGAC</em> CTACCAGGGAAGTTGGTAGCT</td>
</tr>
</tbody>
</table>

**Constructed by:** Jesús Beltrán, 2010  
**Purified by:** Jesús Beltrán  
**DNA Location (-20°C):** Box Number # 10  
**Position:** F1 Conc. 20ng/ul  
**Tube labeled as:** pCol Zm-ZISO 208 H/A  
**Strain Location (-80°C):**  
**XL10 Gold:** Box Number: 17  
**Position:** I3  
**Tube labeled as:** pCol Zm-ZISO 208H/A  
**EBP:** Box Number: 17  
**Position:** J6  
**Tube labeled as:** EBP pCol Zm-ZISO 208H/A  
**NOTE:** This mutation does not affect Zm ZISO activity


**Lab Notebook to reference:** Jesús Beltrán, Depositor’s Name: Jesús Beltrán  
**Original clone name (if different):**

**Organism source of gene:** Maize B73  
**Cloning vector used:** pColaDuet-1, Strain: XL10 Gold  
**Vector size:** 3719bp  
**Insert size:** 1,108bp  
**Antibiotic markers:** Kanamycin  
**Restriction enzyme(s) to release insert:** *BamHI* and *SalI*
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<th>Sequence verified: Site of mutation verified</th>
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**WURTZEL LAB**  
**CLONE INFORMATION**

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<td>Clone type: Expression cDNA clone</td>
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**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 241 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *Bam*HI and *Sal*I

Primers used for amplification from ZmZ-ISO are

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<th>fragment size</th>
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<th>5’ Sequence 3’</th>
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<tbody>
<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>GC <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
</tr>
<tr>
<td>1,108</td>
<td></td>
<td>1,108</td>
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<td>ZmZ-ISO-SalI</td>
<td>2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGAAGTTGCTAGCT</td>
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Constructed by: Jesús Beltrán, 2010

Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 10 Position: F3 Conc. 20ng/ul

Tube labeled as: pCol Zm-ZISO 241 H/A

Strain Location (-80°C)

- XL10 Gold: Box Number: 17 Position: I5. tube labeled as: pCol Zm-ZISO 241H/A
- EBP: Box Number: 17 Position: J8. tube labeled as: EBP pCol Zm-ZISO 241H/A

NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán

Original clone name (if different):

- Organism source of gene: Maize B73
- Cloning vector used: pColaDuet-1, Strain: XL10 Gold
- Vector size: 3719bp Insert size: 1,108bp
- Antibiotic markers: Kanamycin
Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Site of mutation verified   Junction verified: yes, in the original clone

TAACTTTAATAAGGAGATATACCATGGGCAGCAGCCATCAACCATCATCAACCACAG
CCAGGATCCTCTCCTCCACAGCTGGCCTCCACCCATTGCGGTACACGGCTTCCCTCC
TTACCACCGCCGTCGACCTCCCCGCGCCGCCGCTCTGCCCCCACTTAACCACACTCC
CGAGCCGCTCCTCCCCCTTCTTCACACCTGTCTCCACCCACGGTCGTCTCCCGCCGTCG
GGTCGGGGAGGCATCGAGCCGAAGAGGGGTGGTGTGAGGACAGGAGACAGATC
TGGCAGAAGGCCGGCTGCTGGTGAGGATTCGCGGCTGTCATGCGAAGCTCAAGGAC
CAGACGCTGCGTGGGCTATTTACTTGTACGCGGATACGCTAGTGCGTGCTCATTG
CGCTGACGTGCTGGATCACTGACGTCGACGACGGTTGGTTGCGCTGGGACATACTAG
TTTTTTCACTAACCGTCTGCATCCGCGTCACTCAGCGTCGTCACATTGCTTTGC
TTTTTTCTTGATATCTGACAGTTGCTCCTCGCTGTGTGCTGATCTTGCTGTGCAAGCC
TTAATTAGCAATGTCGGAACAGGAAATAATCGATCACCACGACATCACCACAGATG
GTGAGGATATGTTGGGCCTCTGCAACTATAGGAATTGGCACAAGCTAAGTTTTCG
TGAGCAGCCCTCTGTCGGAATCTAGTCATAGCCACACTCTCTTTGTTGGTGTGAAATG
TATCAGGGCTGGTGGCAAGAGGATGAGAGGTGCTGGGATGAACTGTTAACGGGAT
TATCACAAGGAGTTCTTTGCCTGTTACCATATGCAATCACAATGTATACCTTGGG
TGCATACCTTGTCACTCCATTTGATGCAAGCATCCAACACTTCCTCGGTAGG
TCGACAGGCTT

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
**WURTZEL LAB**  
**CLONE INFORMATION**

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<th>Entered into database yes/ # 530</th>
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Clone Number/Name: pCol Zm ZISO 253H/A  
Clone type: Expression cDNA clone

**Clone Description:**  
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 253 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *Bam*HI and *Sal*I

Primers used for amplification from ZmZ-ISO are

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<th>fragment size</th>
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<th>5’ Sequence 3’</th>
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<tbody>
<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
</tr>
<tr>
<td>1,108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGAAGTTGAGTAGCT</td>
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</tbody>
</table>

Constructed by: Jesús Beltrán, 2010  
Purified by: Jesús Beltrán  
DNA Location (-20°C) Box Number # 10 Position: F5 Conc. 20ng/ul  
Tube labeled as: pCol Zm-ZISO 253H/A  
Strain Location (-80°C)  
XL10 Gold: Box Number: 17 Position: I7 tube labeled as: pCol Zm-ZISO 253H/A  
EBP: Box Number: 17 Position: J10. tube labeled as: EBP pCol Zm-ZISO 253H/A  
NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán  
Original clone name (if different):  
Organism source of gene: Maize B73  
Cloning vector used: pColaDuet-1, Strain: XL10 Gold  
Vector size: 3719bp Insert size: 1,108bp  
Antibiotic markers: Kanamycin
Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Site of mutation verified
Junction verified: yes, in the original clone

GATCCTACATGCGCTCCAGCTCCGCCTCCACCTTTGCGGCTACACCCGCCTCTCTCTTTCC
TCACCCTCCGTCCACCTCCGCCGCGCCGTCTGCCCCACCTCACTACCCCAATCCGA
GCGCCGCTCCCCCTCTCTCTCACGCGTTCTCTCCACGCTCGTCGCCCGCTGCGGT
CGGGGGAGGACATCGAGCCGAAAGAGGCAGGTTGTTGCGGAGGGAGACGAGTCTGG
CGGAGGCGGCGTTCTGCGTTGCTCGTAGCCTCGGTTCGCACCTCAGTCAAGGACCAG
AGCGTGGCGTCTGAGGCGTTACTTCCTCGCGGAGATAGTACGTTGCGGTGCTGTTGCGCT
CAACGTCATGTAGTAGACCTACCCGAGAAGTGGTGAAGAATAGTGG
GGGAGCGGTGTTACCCGCGTGTGCTGTGCGTGAATTCAGCTGCTTTTAAGCAGTTACT
ACTATTGTATACCTCATAAATCATCGGTATGATGATGTAATCATTGCGAACGGTCCA
GGGAATACGTGGGATTCATGATGCTCTTCTGTCGTCATTTTCGTTCTTTCT
TCTGTATCCATCAACTTCAATCTCTTTGGAAGTTGGCTGCTTGACAGGAAAGCTG
TACACATGAGGAAACAGGAATATCGTATTACCCAGAGGCCACAGATGTTTGG
TCAGGTAAATTGGTGCCTGCTCCACCCAGTGATGATGCTTGCAACTCAGTGTGGTAG
CGCCTCTGTCGGAATCTATGACCGAGCCACCTCCTCTTGCTTTGCTTGGATAAGTGCAGG
AGGCTGTGTGACGCTATATGGGAGCTCTCGAGATCTGAAGAAGAGAACAAGTG
TTATGGCCCTCGCTGCGATCATCGATGAGCGCAGAACTGCCCAAGATTATCAA
CAAGGGAGTCTTCTCGGTTACCATATGTAAGCAATACAAATGTTAACCTTGGGTGAT
ACTTTGCTCATCCATTAGTGCAAGCATCCAGCTACACACTTCCCTGGTAGTGCAGAC
AAGCTTGCAGGCACGCAATA

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
WURTZEL LAB
CLONE INFORMATION

Date Today: 7/12/2010 Entered into database yes/ # 531

Clone Number/Name: pCol Zm ZISO 332H/A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 332 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *Bam*HI and *Sal*I

Primers used for amplification from ZmZ-ISO1 are

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<th>Fragment size</th>
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<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG <em>GGATCC</em> TATGGCCTCCCAGCTCCGCCTC 1,108</td>
</tr>
<tr>
<td>ZmZ-ISO-Sal I</td>
<td>2587</td>
<td>GC <em>GTCGAC</em> CTACCAGGGAAGTTGAGTGTAGCT</td>
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</table>

Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 10 Position: F 7 Conc. 20ng/ul
- Tube labeled as: pCol Zm-ZISO 332 H/A

Strain Location (-80°C)
- XL10 Gold : Box Number: 17 Position: I9. tube labeled as: pCol Zm-ZISO 332H/A
- EBP : Box Number: 19 Position: H2 tube labeled as: EBP pCol Zm-ZISO 332H/A

NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán

Original clone name (if different):

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1, Strain: XL10 Gold
Vector size: 3719bp Insert size: 1,108bp

Antibiotic markers: Kanamycin
Restriction enzyme(s) to release insert: BamHI and SalI

<table>
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<tr>
<th>Sequence verified: Site of mutation verified</th>
<th>Junction verified: yes, in the original clone</th>
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</table>

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 7/12/2010  
**Entered into database yes/# 532**

**Clone Number/Name:** pCol Zm ZISO 354H/A  
**Clone type:** Expression cDNA clone

**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (with transit sequence) was mutated in H 354 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: **BamHI** and **SalI**

Primers used for amplification from ZmZ-ISO1 are

<table>
<thead>
<tr>
<th>fragment size</th>
<th>Number</th>
<th>5’ Sequence 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmZ-ISO-BamHI</td>
<td>2586</td>
<td>CG <strong>GGATCC</strong> TATGGCCTCCCAGCTCCGCCTC</td>
</tr>
<tr>
<td>1,108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmZ-ISO-SalI</td>
<td>2587</td>
<td>GC <strong>GTCGAC</strong> CTACCAGGAAGTTGGTAGCT</td>
</tr>
</tbody>
</table>

**Constructed by:** Jesús Beltrán, 2010  
**Purified by:** Jesús Beltrán

**DNA Location (-20°C) Box Number # 10 Position: F9 Conc. 20ng/ul**

**Strain Location (-80°C)**
- XL10 Gold : Box Number: 17 Position: J2. tube labeled as: pCol Zm-ZISO 354 H/A
- EBP : Box Number: 19 Position: H4. tube labeled as: EBP pCol Zm-ZISO 354H/A

**NOTE:** This mutation does not affects Zm ZISO activity


**Lab Notebook to reference:** Jesús Beltrán, Depositor’s Name: Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** Maize B73

**Cloning vector used:** pColaDuet-1, Strain: XL10 Gold  
**Vector size:** 3719bp **Insert size:** 1,108bp

**Antibiotic markers:** Kanamycin
<table>
<thead>
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<th>Restriction enzyme(s) to release insert: BamHI and SalI</th>
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</thead>
<tbody>
<tr>
<td>Sequence verified: Site of mutation verified Junction verified: yes, in the original clone</td>
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<tr>
<td>CTTTAATAAGGAGATATACCATTGGGCAGCACGCATCACCATCATCACCACAGCCA</td>
</tr>
<tr>
<td>GGCACCTATGCCCTCCCCAGCTCCGCCTCCACCACCATACCCACAGCAGC</td>
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<td>GCTGTAGTCATAGTGATTGGCATGCGAAGCTACGGGAAAGTGGTGAGAAAATAGTGG</td>
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<td>GGGAGCGGTGTTTACCGTGTGCTGGTCTGGCTGGAATTTCACTGCCTTTAGCAGTTACT</td>
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<td>ACTATTGTACTCTCATAAAATCATCCTGATGATGATGATCTCAAATTATGCGCAGTTCA</td>
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<td>GGGAAATCAGTCGACATTGTCAGCTCTTTTGATGTTCTCCTGCTGTTTACCTTTGCTTTT</td>
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<td>TCTGTAATTCATCCACTTTCAATCTCTTGGAAAGTGTTGGGACAGCTGTTGGGACAGCTAATT</td>
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<td>ACTTTGCTGGGCCATTTGATGCAAGCATCCACGCTACCACACTTCTCCCTGGTAG</td>
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<tr>
<td>NOTES: Conditions for expression are essential as reported by Chen et al., 2010.</td>
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<tr>
<td>Underline seq.: His tag</td>
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</table>
**Clone Number/Name:** pTnT_Zm-ZISO_NTP_Strep  
**Clone type:** For protein *in vitro* translation  
**Depositor’s Name:** Jesús Beltrán

**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73, (Without transit sequence) was cloned in the vector pTnT and formed a strep Tag fusion in the C termini. Restriction sites: *Eco RI* and *Xba I*.

Primers used for amplification from ZmZ-ISO_NTP are:

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<thead>
<tr>
<th>fragment size</th>
<th>Number</th>
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<th>3’</th>
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<td>cgGAATTCTatgcacgtcgtccgcccgtgcgtgcgtgcgtgcgtgcgtgcgtgc</td>
<td>1033</td>
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<td>GAATTC: <em>Eco RI</em> site</td>
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<td>3018</td>
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<td>TCTAGA: <em>Xba I</em> site</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Constructed by:** Jesús Beltrán and Regina Monaco  
**Purified by:** Jesús Beltrán  
**DNA Location (-20°C) Box Number # 10 Position: E7 Conc. 475 ng/ul**  
Tube labeled as: pTnT_Zm-ZISO_NTP_Strep  
**Strain Location (-80°C) Box Number: 17 Position: F2 tube labeled as: pTnT_Zm-ZISO_NTP_Strep.**


**Lab Notebook to reference:** Jesús Beltrán (#1, pag 74) and Regina Monaco  
**Original clone name (if different):**

**Organism source of gene:** Maize B73  
**Cloning vector used:** pTnT, strain: XL10-Gold  
**Vector size:** Insert size:  
**Antibiotic markers:** Ampicillin 50 mg/ml
Restriction enzyme(s) to release insert: *Eco RI* and *Xba I*.

Sequence verified: Yes  
Junction verified: Yes

| TAGAATAACAAGCTACTTGTGTTTTTGCACTCGAGAATTCTATA | GCCTGCGGCTCGGAGGAGGACGATAGCGCCGAGATCCGGAAGGGAGGGTCGTTGTTGCAGGAGGA
| GACGGAGTCTGGCGAGGGCCCGGTGCTCGTGGGGTGAGATTTCGAGTGTCGTGCTGGAATTCG |
| TCAAGGACCAGCAGCTGGCTGCTTGACTTTTACGCCTCCGGGATATAGGCTGCGGTT |
| GCTCGGTGCTGCTCACACGTGCTGATCAGCCCCAGTGACCGGAGGGTCTGACCTGACAAA |
| TCGCTCGAGCTGCTCGCTGCCGACCAGCAGCGAGTTGTTATATGTGCTCCT|
| TACCATAAATTTGCTGTATGTCCATAGTGGTATGCGAAGCTACGGGAAAGTG |
| GAGAAAAATAGTGGGGAGCGTGTCTTACCCTGCTTGCTGTCTGAGTTTCAACACTG |
| CGGTTTGCAGTAATCCATATCTATGGTTGATATCTCCTCATAATAATCATCGGTATGAGTTGACTCAA |
| TATGGCAAGTGTTTCAGGGAATCAGGACTGCATAGCTGTCTTGTGTTATCGTCTGTT |
| CATTTCGTTCTCTTCTGTATCCATCCACATTITCTCTTGGAATTGAGAAGTCAGGT |
| GGACAAACCTAATTACACATGTGGGAAACACAGGAAATAATCGATACACAGACATA |
| CCAACAGTGTTGGGCTAGCTGATCGCATGCTGAGGACGAGGACGAACTG |
| CTCAGTGCTGAGGCTGCTGAGGACTTATCGACCACCATCTCTTGTGAGTT |
| GGAATGGTGAAGAGGCTGTTGTCACGCTATGGTGAAGCCTTGAAGTACTGAA |
| GAAGAGAGAAAGAGGTATGCTGCTGATCGGACGACGGCAGAAGCTT |
| CCCAAAGATTATCACAAGGAGTCTTCTTCTGTTACCATATGATGCAATCACAATG |
| AACCTTTGGTGTCATACCTGCTCATCCATTTGATCGAAAGCTACGCTACCAACATC |
| CTTGATGGGCTGTACATTCACATTGGAAAAATAATTAGAGTACCCCAGGGCGGCGGCG |
| CAAAAAAAAAAACATAGCATAAACCCCTTGGGGGCTC |
| TAAACCGGGTCTTGGAGGGTTTTTTTGGCATCGGGGCTGGCTAATAGCGAAAAAGGCC |
| CGCAGCGATCGCCCTTCCCAACAGATGTCGAGGNOTES: |
| Underline seq.: Strep tag |

**ATG, TAA**: Initiation and termination codons respectively.
**WURTZEL LAB**

**CLONE INFORMATION**

<table>
<thead>
<tr>
<th>Date Today:  7/12/2010</th>
<th>Entered into database yes/ No</th>
<th>580</th>
</tr>
</thead>
</table>

**Clone Number/Name:** PST-Z-ISO (TEE & Hist6)

**Clone type:** Expression cDNA clone

**Clone Description:**

From Hirochi: An NdeI site in Z-ISO was changed from 'catag (HM)' to 'cacatg (HM)' for cloning. (indicated by red letter in the sequence). About another part of the plasmid, please refer to my paper (Kobayashi AEM 2009).

**Primers used:**

<table>
<thead>
<tr>
<th>Number</th>
<th>5’ Sequence</th>
<th>3’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

**fragment size**

**Constructed by:** Hiroshi Kobayashi

**Purified by:** Hiroshi Kobayashi

**DNA Location (-20°C)**

**Tube labeled as:**

**Strain Location (-80°C) Box Number:** 19, 12

**Tube labeled as:** Zm-ZISO PST Bl21 (DE3)

**NOTE:** See supplemental material printed in clone book: induction conditions and original paper.


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized. database # 516

**Cloning vector used:**

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**
143
Sequence verified: Sequence was provided by Hiroshi Kobayashi Junction verified:
PST-Z-ISO(TEE&His6)
ATGAATCATAAAGTGCATCATCATCATCATCACGCAAATATTACCGTTTTCTATAA
CGAAGACTTCCAGGGTAAGCAGGTCGATCTGCCGCCTGGCAACTATACCCGCGCC
CAGTTGGCGGCGCTGGGCATCGAGAATAATACCATCAGCTCGGTGAAGGTGCCGC
CTGGCGTGAAGGCTATCCTGTACCAGAACGATGGTTTCGCCGGCGACCAGATCGA
AGTGGTGGCCAATGCCGAGGAGTTGGGCCCGCTGAATAATAACGTCTCCAGCATC
CGCGTCATCTCCGTGCCCGTGCAGCCGCGCATGGCAAATATTACCGTTTTCTATAA
CGAAGACTTCCAGGGTAAGCAGGTCGATCTGCCGCCTGGCAACTATACCCGCGCC
CAGTTGGCGGCGCTGGGCATCGAGAATAATACCATCAGCTCGGTGAAGGTGCCGC
CTGGCGTGAAGGCTATCCTCTACCAGAACGATGGTTTCGCCGGCGACCAGATCGA
AGTGGTGGCCAATGCCGAGGAGCTGGGTCCGCTGAATAATAACGTCTCCAGCATC
CGCGTCATCTCCGTGCCGGTGCAGCCGAGGGGTACCATTGAAGGCCGCCATatggcga
gccagctgcgtctgcatctggcggcgaccccgccgctgctgccgcatcgtcgtccgcatctgccgcgtccgctgtgcccgaccctgaa
cccgattcgtgcgccgctgccgccgctgagccgtgtgctgagccatgcgcgtccggcgcgtgcggtgggcggcggcattgaaccga
aagaaggcgtggtggcggaaggcgatgaaagcggcggcggcccggtgctggtgggcgaagatagcgcggcgtttgaactgaaag
atcagagcgtggcgagctgggcgtattttgcgggcattctgggcgcggtgctggtggcgctgaacgtgctgtggattgatccgagcac
cggcgtgggcaccaaatttctggatgcggtggcgagcgtgagcgatagccatgaagtggtgatgctgctgctgaccattatttttgcggt
ggtgcatagcggcatggcgagcctgcgtgaaagcggcgaaaaaattgtgggcgaacgtgtgtatcgtgtgctgtttgcgggcattagc
ctgccgctggcggtgaccaccattgtgtattttattaaccatcgttatgatggcacccagctgtggcaggtgcagggcattaccggcattc
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gccgttatggcgaagcgtttgaagtgctgaaaaaacgtaccagcgtgatgccgtttgcggcgattattgatggccgtcagaaactgccg
aaagattatcataaagaattttttcgtctgccgtatgtggcgattaccatgctgaccctgggcgcgtattttgcgcatccgctgatgcaggcg
agcagctatcagctgccgtggtaa
Sequence annotation:
TEE & His6
ATGAATCATAAAGTGCATCATCATCATCATCAC
PST-tag
GCAAATATTACCGTTTTCTATAACGAAGACTTCCAGGGTAAGCAGGTCGATCTGC
CGCCTGGCAACTATACCCGCGCCCAGTTGGCGGCGCTGGGCATCGAGAATAATAC
CATCAGCTCGGTGAAGGTGCCGCCTGGCGTGAAGGCTATCCTGTACCAGAACGAT
GGTTTCGCCGGCGACCAGATCGAAGTGGTGGCCAATGCCGAGGAGTTGGGCCCGC
TGAATAATAACGTCTCCAGCATCCGCGTCATCTCCGTGCCCGTGCAGCCGCGCAT
GGCAAATATTACCGTTTTCTATAACGAAGACTTCCAGGGTAAGCAGGTCGATCTG
CCGCCTGGCAACTATACCCGCGCCCAGTTGGCGGCGCTGGGCATCGAGAATAATA
CCATCAGCTCGGTGAAGGTGCCGCCTGGCGTGAAGGCTATCCTCTACCAGAACGA
TGGTTTCGCCGGCGACCAGATCGAAGTGGTGGCCAATGCCGAGGAGCTGGGTCCG
CTGAATAATAACGTCTCCAGCATCCGCGTCATCTCCGTGCCGGTGCAGCCGAGG
Factor Xa
GGTACCATTGAAGGCCGCCAT
Z-ISO
atggcgagccagctgcgtctgcatctggcggcgaccccgccgctgctgccgcatcgtcgtccgcatctgccgcgtccgctgtgcccga
143


Depositor’s Name: Jesús Beltrán
WURTZEL LAB
CLONE INFORMATION

<table>
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<th>Date Today:  7/12/2010</th>
<th>Entered into database yes/ No_581</th>
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</thead>
<tbody>
<tr>
<td>Clone Number/Name: MCSG9 Z-ISO E1 Brian: “Clone E1 (which I believe you referred to as 79.3-it's predicted molecular weight) is ZmZ-ISO, minus the transit sequence. It begins with the Histidine at AA position 47”. Clone type; Expression cDNA clone</td>
<td></td>
</tr>
</tbody>
</table>

**Clone Description:**
From Brian: The vector places a 10X His tag, in addition to MBP, on the N-terminus. Primers used for amplification from ZmZ-ISO1 are: information not available

<table>
<thead>
<tr>
<th>fragment size</th>
</tr>
</thead>
</table>

**Constructed by:** Brian Kloss, Ph.D.
Senior Research Associate, NYCOMPS
NYSBC
Park Building
89 Convent Avenue
New York, NY 10027

**Purified by:**
DNA Location (-20°C)
Tube labeled as:
Strain Location (-80°C) Box 19, H6
Tube labeled as: MCSG9 Z-ISO E1, strain : BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán
Original clone name (if different):
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516
Expression vector tailored for large-scale, high-throughput purification of recombinant proteins. Protein Expr. Purif. 2006, 47, 446–454.

<table>
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<th>Vector size:</th>
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<tbody>
<tr>
<td>Antibiotic markers: Ampicillin and Chloramphenicol.</td>
</tr>
<tr>
<td>Restriction enzyme(s) to release insert: BamHI and SalI</td>
</tr>
<tr>
<td>Sequence verified: NO Junction verified: NO</td>
</tr>
<tr>
<td>Sequence Not available yet. But will be sequenced.</td>
</tr>
</tbody>
</table>

Depositor’s Name: Jesús Beltrán
pMCSG9
6405 bp
Notes from Dr. Brian Kloss:
I’ve attached a pdf of the map for plasmid pMCSG9. It is the vector into which I have cloned the ZmZ-ISO constructs. Please note that the map indicates that the purification tag is six histidines long. I have converted it to a 10x histidine tag, which allows you to wash a little more stringently to remove any contaminants.

The two glycerol stocks I sent are in T1/T5-resistant BL21(DE3)pLysS. They are Ampicillin (Carbenicillin) and Chloramphenicol-resistant.

I have sent 20ul of each of the two plasmids. The DNA concentrations are written on the side. In case they're not clear, the concentration of clone E1 is 176ng/ul and clone E2 is 108ng/ul.

Clone E1 (which I believe you referred to as 79.3-it's predicted molecular weight) is ZmZ-ISO, minus the transit sequence. It begins with the Histidine at AA position 47. Clone E2 (aka 79.1) is two amino acids shorter and begins with the Arginine at AA position 49.

I'm not sure whether you'll need it, or not, but I have also attached a copy of our large scale (meaning 0.5L culture, or greater) protein purification method.

For protein expression, we use a glycerol stock to inoculate a culture in 2xTY containing Carb. and Chlor. Shake overnight at 37C. Terrific Broth is OK, too. But I would not recommend using LB. We typically pre-warm our media for the next day's expression at 37C. We also include antifoam (Antifoam 204 from Sigma) in all our media.

The next morning, we use 10ml of the overnight culture to inoculate each 500ml 2xTY, plus antibiotics, in a 2L baffle flask. Cultures are shaken at 37C to an A600 of 1.0 (approx. two hours). We then induce protein expression by the addition of IPTG to a final concentration of 1mM. The cells are returned to the 37C shaker for four hours. Harvest cells by centrifugation and store cell pellets in 50ml Falcon tubes at -80C.
WURTZEL LAB
CLONE INFORMATION

Date Today: 04/10/2012 Entered into database yes/No_646

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<th>Clone Number/Name: pMCSG9</th>
<th>Contains MBP. Adds N-terminal His tag, MBP, TEV.</th>
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</thead>
<tbody>
<tr>
<td>Clone type:</td>
<td>Expression cDNA clone</td>
</tr>
</tbody>
</table>

**Clone Description:**
- **Brian:** The vector places a 10X His-FLAG-TEV for protein purification.
- Primers used for amplification
  - Number 5’ Sequence 3’

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location (-20°C):** 10, J7
- **Tube labeled as:** Zm Z-ISO A1
- **Strain Location (-80°C):** 20, F1
- **Tube labeled as:** MCS9 10x His tag : BL21 (DE3)

**NOTE:**

**Cited in journal:**
- Lab Notebook to reference: Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:**

**Cloning vector used:** none

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

<table>
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<tr>
<th>Date Today: 7/12/2010</th>
<th>Entered into database yes/ #647</th>
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</thead>
</table>

Clone Number/Name: pCol Zm Z-ISO NTP 294 D/A  
Clone type: Expression cDNA clone  

**Clone Description:**
ZmZ-ISO1.1 (Data sheet #579), CDS from maize B73 (without transit sequence) was mutated in D 294 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

<table>
<thead>
<tr>
<th>fragment size</th>
<th>Number</th>
<th>5’ Sequence 3’</th>
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<tbody>
<tr>
<td>984bp</td>
<td>3028</td>
<td>cgggatctcacgtcgctcggcgcggcggcgccgctgcg</td>
</tr>
<tr>
<td>2587</td>
<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGAGTAGCT</td>
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</table>

Constructed by: Jesús Beltrán, 2010  
Purified by: Jesús Beltrán  
DNA Location (-20°C) Box Number # 10 Position: J8 Conc. 20ng/ul  
Tube labeled as: pCol Zm-ZISO 294 D/A  
Strain Location (-80°C)  
XL10 Gold: Box Number: 20 Position: F4. tube labeled as: pCol Zm-ZISO 294 D/A  
EBP: Box Number: 22 Position: F6. tube labeled as: EBP pCol Zm-ZISO 294 D/A  
NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán  
Original clone name (if different):

Organism source of gene: Maize B73  
Cloning vector used: pColaDuet-1, Strain: XL10 Gold  
Vector size: 3719bp Insert size:  
Antibiotic markers: Kanamycin  
Restriction enzyme(s) to release insert: BamHI and SalI
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<table>
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<tr>
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<tbody>
<tr>
<td>Sequence verified: Site of mutation verified</td>
<td>Junction verified: yes, in the original clone</td>
</tr>
<tr>
<td><strong>NOTES:</strong> Conditions for expression are essential as reported by Chen et al., 2010.</td>
<td><strong>Underline:</strong></td>
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**WURTZEL LAB**
**CLONE INFORMATION**

Date Today: 7/12/2010          Entered into database yes/ # 779

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<th>pCol Zm Z-ISO NTP 301Y/A</th>
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**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y 301 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

<table>
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<th>fragment size</th>
<th>Number</th>
<th>5’ Sequence 3’</th>
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<td>3028</td>
<td>cggatatctacaGctcgctccccggtccggtcgc</td>
</tr>
<tr>
<td></td>
<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGGTGACT</td>
</tr>
</tbody>
</table>

Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 12 Position: A1 Conc. 20ng/ul
Tube labeled as: pCol Zm-ZISO 301 Y/A
Strain Location (-80°C)
- XL10 Gold : Box Number: 22 Position: A9. tube labeled as: pCol Zm-ZISO 301Y/A
- EBP : Box Number: 22 Position: A10. tube labeled as: EBP pCol Zm-ZISO 301Y/A

NOTE: This mutation does not affects Zm ZISO activity


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán
Original clone name (if different): 

Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1, Strain: XL10 Gold
Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: *BamHI* and *SalI*
Sequence verified: Site of mutation verified  Junction verified: yes, in the original clone

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gccatcaccacacttcaccacacaggatcttacgctgctctcccttatgcgactcctgcctgcattaggaaattaatacgactctaataggggaatgtgacgcgataaaactctccccttgtagaaataattttgtagaaactttttaataaggagatataactcggacagccatcaccacacttcaccacacaggatcttacgctgctctcccttatgcgactcctgcctgcattaggaaattaatacgactctaataggggaatgtgacgcgataaaactctccccttgtagaaataattttgtagaaactttttaataaggagatataactcggacagccatcaccacacttcaccacacaggatcttacgctgctctcccttatgcgactcctgcctgcattaggaaattaatacgactctaataggggaatgtgacgcgataaaactctccccttgtagaaataattttgtagaaactttttaataaggagatataactcggacagccatcaccacacttcaccacacaggatcttacgctgctctcccttatgcgactcctgcctgcattaggaaattaatacgactc

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline: His tag, codon for A # 48(gct) . And Stop codon (tag)
**WURTZEL LAB**
**CLONE INFORMATION**

**Date Today:** 7/12/2010  **Entered into database yes/ # 780**

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**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in C 263 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

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**Number**

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<tr>
<td>Constructed by: Jesús Beltrán, 2010</td>
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<tr>
<td>Purified by: Jesús Beltrán</td>
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<td>Tube labeled as: pCol Zm-ZISO 263 C/A</td>
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<td>Strain Location (-80°C)</td>
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**NOTE:** This mutation does not affect Zm ZISO activity


**Lab Notebook to reference:** Jesús Beltrán, Depositor’s Name: Jesús Beltrán

**Original clone name (if different):**

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<tr>
<td>Organism source of gene: Maize B73</td>
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<tr>
<td>Vector size: 3719bp Insert size:</td>
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<td>Antibiotic markers: Kanamycin</td>
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154
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<td>NOTES: Conditions for expression are essential as reported by Chen et al., 2010. Underline seq.:</td>
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**WURTZEL LAB**
**CLONE INFORMATION**

Date Today: 7/12/2010  Entered into database yes/ #781

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**Clone Description:**

ZmZ-ISO1.1 (Data sheet #579), CDS from maize B73 (without transit sequence) was mutated in E 136 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

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<tr>
<td>2587</td>
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Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán
DNA Location (-20°C) Box Number #12 Position: A3 Conc. 20ng/ul
Tube labeled as: pCol Zm-ZISO 263 C/A
Strain Location (-80°C)
XL10 Gold: Box Number: 22 Position: B3. Tube labeled as: pCol Zm-ZISO 136 E/A
EBP: Box Number: 22 Position: B4. Tube labeled as: EBP pCol Zm-ZISO !36 E/A

**NOTE:**


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1, Strain: XL10 Gold
Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI
Sequence verified: Site of mutation verified  Junction verified: yes, in the original clone

| GAAATTAATACGACTCATTATAGGGGAATTGTGACGGGATAACAAATTCCTCCTGTA    | Underline seq.: His tag |
| GAAATAATTGTGGTTCCTTAATTAAAGGAGATATACCATGGGCGAGCCAGCCATCAC    |                       |
| CATCACCACACACAGCCAGACATCTACTACTCTCTGTCGCTCCCCGGCCGTGCGGGGAGG  |                       |
| GCATCGAGGGAGGGGAGGCTTGTGGCAGGAGGAGACAGTAGTCTGGCGAGGGGC        |
| CCGTGCCTGGGGTGAGATTGGCTCGTTGAGCTCAAGGACAGAAAGCTGGGC          |
| GTCGTCGAGGGATTATTAGCTGCGGGACTCTGACACCTATTTGCTCTGTCCTGCTGCGTC |                       |
| TGCTCACGCCCCAGTACCAGGGGTTCGAGACCACATTCTGACGTCTGTGCCCTCT      |
| CGTCTCCCGACAGCCACGGTTTTATGTTGCTCCTTACCCAAATTGTTTGCTGTAGT     |
| CCATAGTGCATTGCGAAACCTACGGGAAAGTGGTAGGAGAGCAGTAGTGGGGAGAGCG   |
| TGGTTCACGGGTGCTCATTGCCTGGAAATTCCACTGCTTTTACAGTTACTAGTTGTAT  |
| ATACTTCAT..................

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
**Clone Information**

**Clone Number/Name:** pCol Zm Z-ISO NTP 244 E/A  
**Clone type:** Expression cDNA clone

**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in E 244 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

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**Constructed by:** Jesús Beltrán, 2010  
**Purified by:** Jesús Beltrán

**DNA Location (-20°C) Box Number # 12 Position: A4 Conc. 20ng/ul**

- Tube labeled as: pCol Zm Z-ISO 263 C/A

**Strain Location (-80°C)**

- XL10 Gold: Box Number: 22 Position: B5. tube labeled as: pCol Zm-ZISO 244 E/A
- EBP: Box Number: 22 Position: B6. tube labeled as: EBP pCol Zm-ZISO 244 E/A

**NOTE:**

Sequence verified: Site of mutation verified  Junction verified: yes, in the original clone

……GGCGTTTGTTGCAGAGGAGACAGGAGTCTGCGGAGGACCCGAGTTCTGCTGGGTTTACTGCTGCGGAGTACGCGTCTGCTCCGCTAACAGTGCTGGGATAGTCGACCC
CAGTACCCGGGGACCCCAATTTCTCAGCTGCTGCCTCCGCTTCGACAGCAGCAGAGTTTATGTTGCTCCCTTACCATATTTTTTGCTGAGTTCATAGTGGTAT
GGCAAGCTCTAGGAAAGTGTGGGAAAATAGTGGGGAGGGAGGCTGTTTACCGTGTGCTGTTCGCTGGAATTTCACTGCCTTTAGCAGTTACTACTATTGTAATCTTCAT
AA
ATCATCGGTATATGGTACTCAATTATGCGAAGTCCCGAGAATCATGGCTGCATTCAT
GAGCTCTTGTGTCTGTCTTGCTGTTCAGTTTCTTCTTCTTTCTGTATAATTCTCAT
CCACCTAAATTACATGTCGATCAGAGTCTGGAAGCTGACAGCCTAAATTACACATGTGGGCAACAGG
AATAATGCGTATCACCAGACATCCACAGATGTGTTGCTAGGTAATTGTGCTCTT
GCCCATACACTATGGATTCGCAACTCAAGTGGCGAGGCCTGCTGGATGACTTAT
CAGCCCATCTCTGGGTGCTTTGGGAAATGGTACAGGAGCTGCTGCTGCTGAT
GTGAAGCTTTCAGAAGTGACTGGAAGAGAGAACAAGTGTTGCTGTCCGCTGAT
CATCGATGGGACAGAAAAGCATGCTACAAAGGAGGTGTTCTTCCGCTGCTGAT
CCATATGTTAGCAATCACAATGGATACCTTTTGCTGAGATATTCTTGGTAT
CAACATGCTTACCAACTTACCTCGTGCCTGGAAGCTAAGGTTTACCGTGTGCTGAT
NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: Stop codon (TAG)
**WURTZEL LAB**

**CLONE INFORMATION**

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**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y 351 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

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<tr>
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Constructsed by: Jesús Beltrán, 2010

Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 12 Position: A5 Conc. 20ng/ul Tube labeled as: pCol Zm-ZISO 263 C/A

Strain Location (-80°C) XL10 Gold : Box Number: 22 Position: B7. tube labeled as: pCol Zm-ZISO 351 Y/A EBP : Box Number: 22 Position: B8. tube labeled as: EBP pCol Zm-ZISO 351 Y/A

NOTE:


Lab Notebook to reference: Jesús Beltrán, Depositor’s Name: Jesús Beltrán

Original clone name (if different):

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1, Strain: XL10 Gold Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI
Y301A a 5’3
……….GCAGTTACTACTTATTTGATACTTCATAAAATCATCGGTATGATGGTACTCAA
TTATGGCAAGTTCAAGGAATCTATGGCATTATCTGAGTTTCTTTTGTTCTCAGTTT
CATTTCTTCTTTCTCTGTATCCATCCACTTTCAATCTCTTGGAAGTGGCAGCTGT
GGACAGAAGCTAAATTACACATGTGGGAACAGGAATAATGCATATCACCCAAGACAT
CCACAGATGTTGTCAGTAATTTGGTGCTCGTATCAGCTCCTTGCAGCACACTAGTGAATGGCAGAAACTG
CCCACAGATTATCAGCAAGGAGTTCTTTTCGTTACCATATGTAGCAATCAACCTTC
AACCCTGTTGGTGACGCTTATCCTATCCATGGATGCAAGATCCAGCTACCAACTTC
CCTG TAG GTCAAGAAGCTTTCGGGCGCAATAATGCTTAAGTGAACAGAAGTAA
TGATATTGTACACGGCCCTTTGACCACGACTACATAGTGGGAATT
GTGAGCGGATAACAATCCCACATTATGATATATATTTAAGTATAAGAAGGAGAT
ATACATATGGCAGATCTCANTTGGGATATCGCCGCCAGCAGCTACGTCN
GTACCCCTCGAGTCTGTAAGAAAACCCGGCTTGCTGGAAATTTTGAAGGCCAGCACAT
GGACTCGTCTACTAGCGCGCTAATTAACCTNGGCTGTCGCCACCGCTGAGCAA
TAACTAGCATAACCCCTTCTGGSGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGA
AACCTCAGGCATT
NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: Stop codon (TAG)
Clone Number/Name: pCol Zm ZISO NTP 193 Y/A  
Clone type: Expression cDNA clone

**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y 193 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

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<td>3028</td>
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<td>cgggatctcacgctcgcgcccgtgcg</td>
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**Number** | **5’ Sequence 3’**
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3028 | cgggatctcacgctcgcgcccgtgcg 984bp
2587 | GC GTCGAC CTACCAGGGAAGTTGGTAGCT

**Constructed by:** Jesú s Beltrán, 2010  
**Purified by:** Jesú s Beltrán

**DNA Location (-20°C)** Box Number # 12 Position: A6 Conc. 20ng/ul  
Tube labeled as: pCol Zm-ZISO 193Y/A

**Strain Location (-80°C)**  
XL10 Gold : Box Number: 22 Position: B7. tube labeled as: pCol Zm-ZISO 193 Y/A  
EBP : Box Number: 22 Position: B8. tube labeled as: EBP pCol Zm-ZISO 193 Y/A

**NOTE:**

Lab Notebook to reference: Jesú s Beltrán, Depositor’s Name: Jesú s Beltrán

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1, Strain: XL10 Gold  
Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI
Sequence verified: Site of mutation verified | Junction verified: yes, in the original clone

| TACTATTGTCTACTTCCATAAAATCATCGGGCGTGATGGTACTCAATTATGGCAAGTTCTAGGGAATCACTGGCATTCATGGTTCATTTCTCTTTGTTCTCCTCATTCTCTTTTCGAGCTCTCTTCCAATTCATGTTCTCTCTTTGGGAAGTGGCAGATTGCAAAATGCTG
| GTCAGGTAATTTTGTTGCCTTGCCATACACTATGGATTGCAACTCATGTTGACG
| GCGCCCTCTGTGCGGACTTTATCAAGCAGCTCAGCTCTTCTTTGTTCTTGGAAATATTGGAAGTAGACAG
| GAGGCTGTTTGTCACTTGATGTGTTAGCTTCCGGAGTAACTGAAAGAGAAAGATC
| GGTATGGCCTCTCGCTGCTGATGGAACGGAGAGGAATGGCCCCAAGATTATGCA
| ACAAGGAGTTTCTTCTGGGTACCATATGATGCAATCTCAATGATGTTAATGCTGGGCG
| TACTTTGCTCATCCATTGAGCAAGCATCCAGCTACAATTTCCTGGTGGTAGAT
| CAGCGTTGCGGCGGACTACAATGTTAAGTGAACAGAAGGAATGCTATTGTAATAGGCAAC
| CGCCCGACTAATCGGAAATATTACGACTTC

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seqs.: Y193A (GCT) and Stop codon (TAG)
Clone Number/Name: pCol Zm ZISO NTP Y187A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y 187 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<thead>
<tr>
<th>Number</th>
<th>5’ Sequence 3’</th>
<th>fragment size</th>
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<tbody>
<tr>
<td>3028</td>
<td>cgggatctcagctcgctccgcccgtgcg</td>
<td>984bp</td>
</tr>
<tr>
<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGGTAGCT</td>
<td></td>
</tr>
</tbody>
</table>

Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán
DNA Location (-20ºC) Box Number # 12 Position: A7 Conc. 20ng/ul Tube labeled as: pCol Zm-ZISO 187 Y/A
Strain Location (-80ºC) XL10 Gold: Box Number: 22 Position: C1. tube labeled as: pCol Zm-ZISO 187 Y/A EBP: Box Number: 22 Position: C2. tube labeled as: EBP pCol Zm-ZISO 187 Y/A

NOTE: This truncated version is functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Maize B73
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<thead>
<tr>
<th>Cloning vector used: pColaDuet-1</th>
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<tbody>
<tr>
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<tr>
<td>Antibiotic markers: Kanamycin</td>
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<tr>
<td>Restriction enzyme(s) to release insert: BamHI and SalI</td>
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<td>Sequence verified: Yes Junction verified: Yes</td>
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ggcacggttgggagggacgagatctggcggggggcgtgtctctgggtaggtcgggtgccgctctcgcgagctcaaggaac
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NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
Clone Number/Name: pCol Zm Z-ISO NTP Y340A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y340 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are:

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<th>5’ Sequence</th>
<th>3’</th>
</tr>
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<td>984bp</td>
</tr>
<tr>
<td>2587</td>
<td>GC GTCGAC CTACCAGGAAGTTGGTAGCT</td>
<td></td>
</tr>
</tbody>
</table>

Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán
DNA Location (-20°C) Box Number # 12 Position: A8 Conc. 20ng/ul
Tube labeled as: pCol Zm-ZISO 340 Y/A
Strain Location (-80°C)
XL10 Gold: Box Number: 22 Position: C3. tube labeled as: pCol Zm-Z-ISO 340 Y/A
EBP: Box Number: 22 Position: C4. tube labeled as: EBP pCol ZmZ-ISO 340 Y/A

NOTE:

NOTE: This truncated version is functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1
Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Yes Junction verified:

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| GGGTGCGGAGGAGCATCGAGCCGAGAGAGAGGCGTTTTGCGAGGAGGAGACAGAG | TCTGGCGAGAGGCCCCGTCTCGTGGGTAGAGATTCCGCTCGTCTCGAGCTCAAGG |
| ACCAGAGCGCTGGCTGTCGTTACTTCCACGGGATACTAGTTGCAGTGCTGCTGCTG | TGCAGCTCAACGCTGCTGAGATCGACCCAGTACCGGGGACCCAGCTGCGGAGC |
| GACGCCGTCTCGTCCGCCAGACGCCAGGTGTATGTGGCTCTTACCATTAAT | AAATTGTCTGTAGTCCATAGGGCTATGGGGGGAAGTGCTGAGAGAA |
| ATAGTGAGGCAGCTGGTTTACCCGCTGCTGGTTCGCTGGAATTTCACTGCTTTTAGC | AGTTACTACTATTGATACATACATCCACATCGGTATATGTGTAATCATTAGG |
| AGTATTACATACATACATACATACATACATACATCGGTATATGTGTATACATTAGC | AGATTTCAAGGAAATCACTGGCAGCTCGAATACGTGGACTTATCAGCACCATCTCTTTGGTGCTTGGAATG |
| GTGACAGGAGGTGCTTTGCTACGCTATGGTAGCGTTCCGAAAGTACTGAAAGAG | AACAAGTTATGCTCCCTTGCTGCTGATCGACGGCAAGAACCTGACCACAA |
| GCTAAAAATTACACATGGAAGAGAAAAAATGGCTGATATCCAGAGCATCTCCACAG | GGATTATCCAGGAGTCTTCTCGTTTCAATCCACATCCATCTCTTTGGTGCTTGGAATG |
| TGCCGTAAGCGCGCTCTGTCGAGCTACTAGGACCCACTCTCTTTGGTGCTTGGAATG | GTGACAGGAGGTGCTTTGCTACGCTATGGTAGCGTTCCGAAAGTACTGAAAGAG |
| AACAAGTTATGCTCCCTTGCTGCTGATCGACGGCAAGAACCTGACCACAA |

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
Clone Number/Name: pCol Zm Z-ISO NTP Y223A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y223 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<tr>
<td></td>
<td>2587</td>
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</table>

BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<th>Number</th>
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<tbody>
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<td>3028</td>
<td>cgggatcctcacgctcgtcccgcccgtgcg</td>
</tr>
<tr>
<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGGTAGCT</td>
</tr>
</tbody>
</table>

Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán
DNA Location (-20°C) Box Number # 12 Position: A9 Conc. 20ng/ul Tube labeled as: pCol Zm-ZISO 223 Y/A
Strain Location (-80°C)
XL10 Gold : Box Number: 22 Position: C5. tube labeled as: pCol Zm Z-ISO 223 Y/A EBP : Box Number: 22 Position: C6. tube labeled as: EBP pCol ZmZ-ISO 223 Y/A

NOTE:

NOTE: This truncated version is functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1  
Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Yes  
Junction verified:

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<tr>
<td>NOTEnotes: Conditions for expression are essential as reported by Chen et al., 2010.</td>
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</table>

Underline seq.:
**WURTZEL LAB**
**CLONE INFORMATION**

**Date Today:** 7/12/2010

**Entered into database yes/ No_**

**788**

<table>
<thead>
<tr>
<th>Clone Number/Name: pCol Zm Z-ISO NTP D330A</th>
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<tbody>
<tr>
<td>Clone type: Expression cDNA clone</td>
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<tr>
<td><strong>Clone Description:</strong></td>
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<tr>
<td>ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in D 330 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: <em>BamHI</em> and <em>SalI</em></td>
</tr>
<tr>
<td>Primers used for amplification from ZmZ-ISO1 are</td>
</tr>
<tr>
<td><strong>Number</strong></td>
</tr>
<tr>
<td>fragment size</td>
</tr>
<tr>
<td>3028</td>
</tr>
<tr>
<td>2587</td>
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<tr>
<td>Constructed by: Jesús Beltrán, 2010</td>
</tr>
<tr>
<td>Purified by: Jesús Beltrán</td>
</tr>
<tr>
<td>DNA Location (-20°C) Box Number # 12 Position: A10 Conc. 20ng/ul</td>
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<tr>
<td>Tube labeled as: pCol Zm-ZISO 330 D/A</td>
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<tr>
<td>Strain Location (-80°C)</td>
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<tr>
<td>XL10 Gold : Box Number: 22 Position: C7. tube labeled as: pCol Zm Z-ISO 330 D/A</td>
</tr>
<tr>
<td>EBP : Box Number: 22 Position: C8. tube labeled as: EBP pCol ZmZ-ISO 330 D/A</td>
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</table>

**NOTE:**

**NOTE:** This truncated version is functional as demonstrated by complementation in E. coli


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene: Maize B73**
| Cloning vector used: pColaDuet-1 |
| Vector size: 3719bp Insert size: |
| Antibiotic markers: Kanamycin |
| Restriction enzyme(s) to release insert: BamHI and SalI |
| Sequence verified: Yes Junction verified: |

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TGCCCACACTATACATGGATTGGAACCTACGTTGCCGTAAGCGGCTATGTGCTGGAACCTA
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TCATCGATGGACCGCAGAAACTGCCCAGATGCTTATCACAAGGA....
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NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: (GCT) D330A
Clone Number/Name: pCol Zm Z-ISO NTP D294A  
Clone type: Expression cDNA clone  

**Clone Description:**  
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in D294 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*  

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<th>5’ Sequence 3’</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3028</td>
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<td>984bp</td>
</tr>
<tr>
<td>2587</td>
<td>GC GTCGAC CTACCAGGAAGTGGTCTA</td>
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</table>

Primers used for amplification from ZmZ-ISO1 are  

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<th>5’ Sequence 3’</th>
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<tbody>
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<td>cgggatctcgctcgctcggcccgtg</td>
</tr>
<tr>
<td>2587</td>
<td>GC GTCGAC CTACCAGGAAGTGGTCTA</td>
</tr>
</tbody>
</table>

Constructed by: Jesús Beltrán, 2013  
Purified by: Jesús Beltrán  
DNA Location (-20°C) Box Number # 12 Position: B7 Conc. 20ng/ul  
Tube labeled as: pCol Zm-ZISO NTP 294 D/A  
Strain Location (-80°C)  
XL10 Gold: Box Number: 22 Position: D6.  tube labeled as: pCol Zm Z-ISO NTP 294 D/A  
EBP Box Number: 22 Position: D7.  tube labeled as: EBP pCol ZmZ-ISO NTP 294 D/A

NOTE: Use this clone instead of clone # 647. This truncated version is functional as demonstrated by complementation in E. coli.


Lab Notebook to reference: Jesús Beltrán  
Original clone name (if different):
Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1
Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Yes Junction verified:

Sequence:

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
Clone Number/Name: pCol Zm Z-ISO NTP C263A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in C263 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<th>5' Sequence 3'</th>
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</thead>
<tbody>
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<td>3028</td>
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<tr>
<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGTTGAGCT</td>
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</table>

fragment size
984bp

Constructed by: Jesús Beltrán, 2013
Purified by: Jesús Beltrán
DNA Location (-20°C) Box Number 12, Position: B8. Conc. 20ng/µl
Tube labeled as: pCol Zm-ZISO NTP 263 C/A
Strain Location (-80°C)
XL10 Gold: Box Number: 22 Position: D8. Tube labeled as: pCol Zm Z-ISO NTP 263 C/A
EBP: Box Number: 22 Position: D9. Tube labeled as: EBP pCol ZmZ-ISO NTP 263 C/A

NOTE: This truncated version is functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1
Vector size: 3719bp Insert size:
Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Yes Junction verified:

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NOTES: Conditions for expression are essential as reported by Chen et al., 2010.

Underline seq.: His tag
Clone Number/Name: pCol Zm Z-ISO NTP H150A
Clone type; Expression cDNA clone

**Clone Description:**
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in H150 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<tr>
<th>Number</th>
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<tr>
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<td>984bp</td>
</tr>
<tr>
<td>2587</td>
<td>GC GTCGAC</td>
<td>CTACCAGGAAGTTGCT</td>
</tr>
</tbody>
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Constructed by: Jesús Beltrán, 2013
Purified by: Jesús Beltrán
DNA Location (-20°C) Box Number # 12 Position: B9 Conc. 20ng/ul
Tube labeled as: pCol Zm Z-ISO NTP 150 H/A
Strain Location (-80°C)
XL10 Gold: Box Number: 22 Position: D10. tube labeled as: pCol Zm Z-ISO NTP 150 H/A
EBP Box Number: 22 Position: E1. tube labeled as: EBP pCol ZmZ-ISO NTP 150 H/A

NOTE: This truncated version affects the activity of Z-ISO as demonstrated by complementation in *E. coli*.


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
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<th><strong>Organism source of gene:</strong> Maize B73</th>
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<td><strong>Cloning vector used:</strong> pColaDuet-1</td>
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<td><strong>Vector size:</strong> 3719bp <strong>Insert size:</strong></td>
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<tr>
<td><strong>Antibiotic markers:</strong> Kanamycin</td>
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<td><strong>Restriction enzyme(s) to release insert:</strong> BamHI and SalI</td>
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<tr>
<td><strong>Sequence verified:</strong> Yes <strong>Junction verified:</strong></td>
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```

**NOTES:** Conditions for expression are essential as reported by Chen et al., 2010. **Underline seq.**: His tag
Clone Number/Name: pCol Zm Z-ISO NTP H266A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in H266 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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Constructed by: Jesús Beltrán, 2013
Purified by: Jesús Beltrán
DNA Location (-20°C) Box Number # 12 Position: B10. Conc. 20ng/ul
Tube labeled as: pCol Zm-ZISO NTP 266 H/A
Strain Location (-80°C)
XL10 Gold: Box Number: 22 Position: E2. tube labeled as: pCol Zm Z-ISO NTP 266 H/A
EBP Box Number: 22 Position: E3. tube labeled as: EBP pCol ZmZ-ISO NTP 266 H/A

NOTE: This clone is not functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
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<td><strong>Insert size:</strong></td>
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<td>BamHI and SalI</td>
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<td><strong>Junction verified:</strong></td>
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**NOTES:** Conditions for expression are essential as reported by Chen et al., 2010.

**Underline seq.:** His tag

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<td>MCSG9 Z-ISO E2</td>
<td><strong>Brief Description:</strong></td>
<td>“Clone E2 (aka 79.1) is two amino acids shorter and begins with the Arginine at AA position 49”</td>
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<tr>
<td><strong>Clone type:</strong></td>
<td>Expression cDNA clone</td>
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**Clone Description:**
From Brian: The vector places a 10X His tag, in addition to MBP, on the N-terminus.

**Constructed by:** Brian Kloss, Ph.D.  
Senior Research Associate, NYCOMPS  
NYSBC  
Park Building  
89 Convent Avenue  
New York, NY 10027  
Purified by:
**DNA Location (-20°C):** 10, G3  
**Tube labeled as:** MCSG9 Z-ISO E2  
**Strain Location (-80°C):** 19, H7 BL21 (DE3), 22, E5 C43 (DE3)  
**Tube labeled as:** MCSG9 Z-ISO E2  
**NOTE:** Use 22, E5 C43 (DE3) for better expression.


**Lab Notebook to reference:** Jesús Beltrán  
**Original clone name (if different):**  
**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516  
**Vector size**
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<td>Restriction enzyme(s) to release insert:</td>
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<td>Sequence verified: NO Junction verified: NO</td>
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<tr>
<td>Sequence Not available yet. But will be sequenced.</td>
</tr>
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Depositor’s Name: Jesús Beltrán
Notes from Dr. Brian Kloss:
I’ve attached a pdf of the map for plasmid pMCSG9. It is the vector into which I have cloned the ZmZ-ISO constructs. Please note that the map indicates that the purification tag is six histidines long. I have converted it to a 10x histidine tag, which allows you to wash a little more stringently to remove any contaminants.

The two glycerol stocks I sent are in T1/T5-resistant BL21(DE3)pLysS. They are Ampicillin (Carbenicillin) and Chloramphenicol-resistant.

I have sent 20ul of each of the two plasmids. The DNA concentrations are written on the side. In case they're not clear, the concentration of clone E1 is 176ng/ul and clone E2 is 108ng/ul.

Clone E1 (which I believe you referred to as 79.3-it's predicted molecular weight) is ZmZ-ISO, minus the transit sequence. It begins with the Histidine at AA position 47. Clone E2 (aka 79.1) is two amino acids shorter and begins with the Arginine at AA position 49.

I'm not sure whether you'll need it, or not, but I have also attached a copy of our large scale (meaning 0.5L culture, or greater) protein purification method.

For protein expression, we use a glycerol stock to inoculate a culture in 2xTY containing Carb. and Chlor. Shake overnight at 37C. Terrific Broth is OK, too. But I would not recommend using LB. We typically pre-warm our media for the next day's expression at 37C. We also include antifoam (Antifoam 204 from Sigma) in all our media.

The next morning, we use 10ml of the overnight culture to inoculate each 500ml 2xTY, plus antibiotics, in a 2L baffle flask. Cultures are shaken at 37C to an A600 of 1.0 (approx. two hours). We then induce protein expression by the addition of IPTG to a final concentration of
1mM. The cells are returned to the 37°C shaker for four hours. Harvest cells by centrifugation and store cell pellets in 50ml Falcon tubes at -80°C.
WURTZEL LAB
CLONE INFORMATION

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**Clone Number/Name:** Zm Z-ISO A1, contains Z-ISO (full length) with a 10X His-FLAG-TEV tag.

**Clone type:** Expression cDNA clone

**Clone Description:**
**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO1 are: information not available

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**Purified by:**

**DNA Location** (-20°C): 11, A1

Tube labeled as: Zm Z-ISO A1

**Strain Location** (-80°C): 21, A1

Tube labeled as: Zm Z-ISO A1, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

| Date Today: | 04/10/2012 | Entered into database: yes/No: 667 |

| Clone Number/Name: | Zm Z-ISO A2, contains Z-ISO (truncation) with a 10X His-FLAG-TEV tag. |
| Clone type: | Expression cDNA clone |

**Clone Description:**
- **Brian:** The vector places a 10X His-FLAG-TEV for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

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**NOTE:**


Lab Notebook to reference: Jesús Beltrán

**Original clone name (if different):**

| Organism source of gene: | A synthetic Z-ISO gene, ACA less and codon optimized: database # 516 |

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

| Antibiotic markers: | Kanamycin |

| Restriction enzyme(s) to release insert: |

**Sequence verified:** Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No

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**Clone Description:**  
**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**
- **DNA Location** (-20°C): 11, A3  
- Tube labeled as: **Zm Z-ISO A3**  
- **Strain Location** (-80°C): 21, A3  
- Tube labeled as: **Zm Z-ISO A3** strain BL21 (DE3)

**NOTE:**
- Lab Notebook to reference: Jesús Beltrán  
- Original clone name (if different):  
- Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database #516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/No_669

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### NOTE:

- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):  
- Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516
- **Cloning vector used:** pNYCOMPS N-Term.
- **Vector size:**  
- **Antibiotic markers:** Kanamycin
- **Restriction enzyme(s) to release insert:**  
- **Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

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**Clone Number/Name:** Zm Z-ISO A5, contains Z-ISO (truncation) with a 10X His-FLAG-TEV tag.

**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

**DNA Location** (-20ºC): 11, A5

Tube labeled as: Zm Z-ISO A5

**Strain Location** (-80ºC): 21, A5

Tube labeled as: Zm Z-ISO A5, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
WURTZEL LAB
CLONE INFORMATION

Date Today: 04/10/2012
Entered into database yes/ No_671

| Clone Number/Name: Zm Z-ISO A6, contains Z-ISO (truncation) with a 10X His-FLAG-TEV tag. |
| Clone type: Expression cDNA clone |

Clone Description:
Brian: The vector places a 10X His-FLAG-TEV for protein purification.
Primers used for amplification from ZmZ-ISO are: information not available

| fragment size |
| Number | 5’ Sequence 3’ |

Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:
DNA Location (-20°C): 11, A6
Tube labeled as: Zm Z-ISO A6
Strain Location (-80°C): 21, A6
Tube labeled as: Zm Z-ISO A6, strain: BL21 (DE3)

NOTE:

Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

Cloning vector used: pNYCOMPS N-Term

Vector size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified
**WURTZEL LAB**

**CLONE INFORMATION**

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**Clone Number/Name:** Zm Z-ISO A7, contains Z-ISO (truncation) with a 10X His-FLAG-TEV tag.

**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, A7

Tube labeled as: Zm Z-ISO A7

**Strain Location** (-80°C) 21, A7

Tube labeled as: Zm Z-ISO A7, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### WURTZEL LAB
#### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No _673_

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**Clone Description:**

- Brian: The vector places a 10X His-FLAG-TEV for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available
  
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**Construct**

- Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

- **DNA Location (-20°C):** 11, A8
- Tube labeled as: Zm Z-ISO A8
- **Strain Location (-80°C) 21, A8**
- Tube labeled as: Zm Z-ISO A8, strain: BL21 (DE3).

**NOTE:**

- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:


| **WURTZEL LAB**  
| **CLONE INFORMATION**  

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No_674

| Clone Number/Name: Zm Z-ISO A9, contains Z-ISO (truncation) with a 10X His-FLAG-TEV tag.  
| **Clone type:** Expression cDNA clone

| **Clone Description:**  
| **Brian:** The vector places a 10X His-FLAG-TEV for protein purification.  
| Primers used for amplification from ZmZ-ISO are: information not available  
| Number  5’ Sequence 3’

| **Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park  
| Building 89 Convent Avenue New York, NY 10027  
| **Purified by:**

| **DNA Location** (-20°C): 11, A9  
| Tube labeled as: Zm Z-ISO A9  
| **Strain Location** (-80°C) 21, A9  
| Tube labeled as: Zm Z-ISO A9, strain: BL21 (DE3)

| **NOTE:**


| **Lab Notebook to reference:** Jesús Beltrán

| **Original clone name (if different):**

| **Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

| **Cloning vector used:** pNYCOMPS N-Term.

| **Vector size:**

| **Antibiotic markers:** Kanamycin

| **Restriction enzyme(s) to release insert:**

| **Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

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**Date Today:** 04/10/2012  
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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

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**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

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**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

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### Clone Description:

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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### Constructed by:

Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

**DNA Location** (-20°C): 11, B2
Tube labeled as: Zm Z-ISO A12

**Strain Location** (-80°C) 21, B2
Tube labeled as: Zm Z-ISO A12, strain: BL21 (DE3)

### NOTE:


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

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**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location (-20°C):** 11, B3

Tube labeled as: Zm Z-ISO B1

**Strain Location (-80°C):** 21, B3

Tube labeled as: Zm Z-ISO B1, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Organism clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012

**Entered into database:** yes/ No_679

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**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Fragment size**

**Built by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, B4

Tube labeled as: Zm Z-ISO B2

**Strain Location** (-80°C): 21, B4

Tube labeled as: Zm Z-ISO B2, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

Date Today:  04/10/2012  
Entered into database yes/ No_680

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**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

**DNA Location** (-20°C) : 11, B5
Tube labeled as: Zm Z-ISO B3

**Strain Location** (-80°C) 21, B5
Tube labeled as: Zm Z-ISO B3, strain : BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

Date Today: 04/10/2012 Entered into database yes/No 681

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**Clone Description:**
Brian: The vector places a 10X His-FLAG-TEV for protein purification.
Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:

DNA Location (-20°C): 11, B5
Tube labeled as: Zm Z-ISO B4
Strain Location (-80°C) 21, B6
Tube labeled as: Zm Z-ISO B4, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

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**Clone type:** Expression cDNA clone

**Clone Description:**  
**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available  
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**Fragment size**

**Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**

**DNA Location** (-20°C) : 11, B7  
Tube labeled as: Zm Z-ISO B5  
**Strain Location** (-80°C) 21, B7  
Tube labeled as: Zm Z-ISO B5, strain: BL21 (DE3)

**NOTE:**

**Lab Notebook to reference:** Jesús Beltrán  
**Original clone name (if different):**

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database** yes/No: 683

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**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available  
Number fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, B8  
Tube labeled as: Zm Z-ISO B6  
**Strain Location** (-80°C): 21, B8  
Tube labeled as: Zm Z-ISO B6, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database #516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
WURTZEL LAB  
CLONE INFORMATION

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| Clone type: Expression cDNA clone |

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| Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027 |
|------------------------|----------------------------------|
| Purified by: |

| DNA Location (-20°C): 11, B9 |
| Tube labeled as: Zm Z-ISO B7 |

| Strain Location (-80°C): 21, B9 |
| Tube labeled as: Zm Z-ISO B7, strain: BL21 (DE3) |

| NOTE: |
|------------------------|----------------------------------|
| Lab Notebook to reference: Jesús Beltrán |

| Original clone name (if different): |
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| Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516 |

| Cloning vector used: pNYCOMPS N-Term. |
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| Vector size: |

| Antibiotic markers: Kanamycin |
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| Restriction enzyme(s) to release insert: |
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| Sequence verified: Junction verified: |
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**WURTZEL LAB**
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database yes/No:** Yes

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**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-FLAG-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location (-20°C):** 11, B10

Tube labeled as: Zm Z-ISO B8

**Strain Location (-80°C):** 21, B10

Tube labeled as: Zm Z-ISO B8, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pNYCOMPS N-Term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
WURTZEL LAB
CLONE INFORMATION

Date Today: 04/10/2012

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**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, C1

Tube labeled as: Zm Z-ISO C1

**Strain Location** (-80°C): 21, C1

Tube labeled as: Zm Z-ISO C1, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

**Date Today: 04/10/2012**  
**Entered into database yes/ No_687**

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**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**fragment size**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park  
Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C) : 11, C2  
Tube labeled as: Zm Z-ISO C2  
**Strain Location** (-80°C) : 21, C2  
Tube labeled as: Zm Z-ISO C2, strain : BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No _688_

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**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location (-20°C):** 11, C3  
Tube labeled as: Zm Z-ISO C3

**Strain Location (-80°C):** 21, C3  
Tube labeled as: Zm Z-ISO C3, strain : BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

Date Today: 04/10/2012  
Entered into database: yes/ No: 689

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**Clone type:** Expression cDNA clone

**Clone Description:**
**Brian:** The vector places a 10X His-TEV for protein purification.
Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:
**DNA Location** (-20°C): 11, C4
Tube labeled as: Zm Z-ISO C4

**Strain Location** (-80°C): 21, C4
Tube labeled as: Zm Z-ISO C4, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
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**Date Today:** 04/10/2012  
**Entered into database:** yes/ No

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**Clone Description:**

- **Brian:** The vector places a 10X His-TEV for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available

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**Fragment size:**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

- **DNA Location (-20°C):** 11, C5
- Tube labeled as: Zm Z-ISO C5
- **Strain Location (-80°C):** 21, C5
- Tube labeled as: Zm Z-ISO C5, strain: BL21 (DE3)

**NOTE:**


- Lab Notebook to reference: Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**Clone Information**

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**Date Today:** 04/10/2012  
**Entered into database:** yes/No 691

### Clone Number/Name

**Zm Z-ISO C6**

Contains Z-ISO (truncation) with a 10X His-TEV tag.

### Clone Description

**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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### Constructed by

Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC  
Building 89 Convent Avenue  
New York, NY 10027

### Purified by

#### DNA Location

(-20°C): 11, C6

Tube labeled as: Zm Z-ISO C6

#### Strain Location

(-80°C): 21, C6

Tube labeled as: Zm Z-ISO C6, strain: BL21 (DE3)

###NOTE:


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database yes/ No:** Yes

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**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Fragment size**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, C7  
Tube labeled as: Zm Z-ISO C7

**Strain Location** (-80°C): 21, C7  
Tube labeled as: Zm Z-ISO C7, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:

Sequence Not available yet.
WURTZEL LAB
CLONE INFORMATION

Date Today: 04/10/2012
Entered into database yes/ No

**Clone Number/Name:** Zm Z-ISO C8, contains Z-ISO (truncation) with a 10X His-TEV tag.  
**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

**DNA Location** (-20°C): 11, C8  
Tube labeled as: Zm Z-ISO C8

**Strain Location** (-80°C): 21, C8  
Tube labeled as: Zm Z-ISO C8, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

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| **Clone Description:**  
Brian: The vector places a 10X His-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available  
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| **Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
Purified by:  
**DNA Location** (-20°C): 11, C10  
Tube labeled as: Zm Z-ISO C10  
**Strain Location** (-80°C): 21, C10  
Tube labeled as: Zm Z-ISO C10, strain: BL21 (DE3) |

**NOTE:**  
Lab Notebook to reference: Jesús Beltrán  
Original clone name (if different):  
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

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| **CLONE INFORMATION** |
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| **Date Today:** 04/10/2012 | **Entered into database:** yes/No_696 |

| **Clone Number/Name:** Zm Z-ISO C11, contains Z-ISO (truncation) with a 10X His-TEV tag. |
| **Clone type:** Expression cDNA clone |

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**NOTE:**


| **Lab Notebook to reference:** Jesús Beltrán |
| **Original clone name (if different):** |

| **Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized : database # 516 |

| **Cloning vector used:** pMCSG7-10x His |
| **Vector size:** |
| **Antibiotic markers:** Ampicillin |

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### CLONE INFORMATION

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**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.  
Primer used for amplification from ZmZ-ISO are: information not available  
Number 5’ Sequence 3’

**constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location (-20°C):** 11, D3  
Tube labeled as: Zm Z-ISO D1  
**Strain Location (-80°C):** 21, D3  
Tube labeled as Zm Z-ISO D1, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

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**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**fragment size**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, D4
Tube labeled as: Zm Z-ISO D2

**Strain Location** (-80°C): 21, D4
Tube labeled as: Zm Z-ISO D2, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database:** yes/No_700

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**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**

**DNA Location** (-20°C): 11, D5  
Tube labeled as: Zm Z-ISO D3

**Strain Location** (-80°C) 21, D5  
Tube labeled as: Zm Z-ISO D3, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

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**Clone type:** Expression cDNA clone

**Clone Description:**  
**Brian:** The vector places a 10X His-TEV for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C) : 11, D6  
Tube labeled as: Zm Z-ISO D4

**Strain Location** (-80°C) 21, D6  
Tube labeled as: Zm Z-ISO D4, strain : BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

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**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, D7

Tube labeled as: Zm Z-ISO D5

**Strain Location** (-80°C) 11, D7

Tube labeled as: Zm Z-ISO D5, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
Clone Number/Name: Zm Z-ISO D6, contains Z-ISO (truncation) with a 10X His-TEV tag.
Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a 10X His-TEV for protein purification.
Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:
DNA Location (-20°C) : 11, D8
Tube labeled as: Zm Z-ISO D6
Strain Location (-80°C) : 21, D8
Tube labeled as: Zm Z-ISO D6, strain : BL21 (DE3)

NOTE:
Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

Cloning vector used: pMCSG7-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No _704

| Clone Number/Name: Zm Z-ISO D7, contains Z-ISO (truncation) with a 10X His-TEV tag. |  
|---|---|
| **Clone type:** Expression cDNA clone |  

**Clone Description:**

**Brian:** The vector places a 10X His-TEV for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, D9

Tube labeled as: Zm Z-ISO D7

**Strain Location** (-80°C): 21, D9

Tube labeled as: Zm Z-ISO D7, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### WURTZEL LAB
#### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No_705

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**Clone Description:**
- **Brian:** The vector places a 10X His-TEV for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available
  - **Number** 5’ Sequence 3’
  - **fragment size**

**Built by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location** (-20°C): 11, D10
  - Tube labeled as: Zm Z-ISO D8
- **Strain Location** (-80°C): 21, D10
  - Tube labeled as: Zm Z-ISO D8, strain: BL21 (DE3)

**NOTE:**
- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- **Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
Clone Number/Name: Zm Z-ISO E1, contains Z-ISO (full length) (minus the transit sequence) with a 10X His-MBP-TEV tag. It begins with the Histidine at AA position 47.

Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a 10X His tag, in addition to MBP, on the N-terminus. Primers used for amplification from ZmZ-ISO are: information not available.

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constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:
DNA Location (-20°C): 11, E1
Tube labeled as: Zm Z-ISO E1

Strain Location (-80°C): 21, E1
Tube labeled as: Zm Z-ISO E1, strain: BL21 (DE3)

NOTE:

Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

Cloning vector used: pMCSG9-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

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**Clone Number/Name:** Zm Z-ISO E2, contains Z-ISO (truncation, begins at R49) with a 10X His-TEV tag. Clone E2 is two amino acids shorter and begins with the Arginine at AA position 49

**Clone type:** Expression cDNA clone

**Clone Description:**
- **Brian:** The vector places a 10X His-TEV for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location** (-20°C): 11, E2
  - Tube labeled as: Zm Z-ISO E2
- **Strain Location** (-80°C)  21, E2
  - Tube labeled as: Zm Z-ISO E2, strain: BL21 (DE3)

**NOTE:**
- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- **Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

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**Clone Number/Name:** Zm Z-ISO E3, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag.

**Clone type:** Expression cDNA clone

**Clone Description:**

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, E3

Tube labeled as: Zm Z-ISO E3

**Strain Location** (-80°C) 21, E3

Tube labeled as: Zm Z-ISO E3, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

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**Clone type:** Expression cDNA clone

**Clone Description:**
- **Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available
  - Number 5’ Sequence 3’

**Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, E4
- Tube labeled as: Zm Z-ISO E4

**Strain Location** (-80°C): 21, E4
- Tube labeled as: Zm Z-ISO E4, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database yes/ No:** Yes

| **Clone Number/Name:** Zm Z-ISO E5, contains Z-ISO (truncation, begins at G55) with a 10X His-MBP-TEV tag. |
| **Clone type:** Expression cDNA clone |

**Clone Description:**

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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fragment size

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

| **DNA Location** (-20°C) | 11, E5 |
| **Strain Location** (-80°C) | 21, E5 |

Tube labeled as: Zm Z-ISO E5

Tube labeled as: Zm Z-ISO E5, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database #516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
Clone Number/Name: Zm Z-ISO E6, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag.
Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a 10X His-MBP-TEV tag for protein purification.
Primers used for amplification from ZmZ-ISO are: information not available

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Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:
DNA Location (-20°C): 11, E6
Tube labeled as: Zm Z-ISO E6
Strain Location (-80°C): 21, E6
Tube labeled as: Zm Z-ISO E6, strain: BL21 (DE3)

NOTE:

Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

Cloning vector used: pMCSG7-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
### WURTZEL LAB
#### CLONE INFORMATION

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**WURTZEL LAB**  
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No_713

| **Clone Number/Name:** Zm Z-ISO E8, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag. |
| **Clone type:** Expression cDNA clone |

**Clone Description:**

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

| Fragment size |
| Number              | 5’ Sequence 3’ |

**Built by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park  
**Building 89 Convent Avenue New York, NY 10027**  
**Purified by:**

**DNA Location** (-20°C): 11, E8  
**Tube labeled as:** Zm Z-ISO E8

**Strain Location** (-80°C): 21, E8  
**Tube labeled as:** Zm Z-ISO E8, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database #516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No_714

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| Constructed by    | Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027 |
| Purified by       | DNA Location (-20°C): 11, E9                                      |
|                   | Tube labeled as: : Zm Z-ISO E9                                   |
|                   | Strain Location (-80°C) 21, E9                                   |
|                   | Tube labeled as: : Zm Z-ISO E9, strain : BL21 (DE3)              |

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### WURTZEL LAB

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database:** yes/No 715

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**Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, E10  
Tube labeled as: Zm Z-ISO E10

**Strain Location** (-80°C) 21, E10  
Tube labeled as: Zm Z-ISO E10, strain: BL21 (DE3)

### NOTE:


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

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**Purified by:**

**DNA Location** (-20°C) : 11, F1  
Tube labeled as: Zm Z-ISO E11

**Strain Location** (-80°C) 21, F1  
Tube labeled as: Zm Z-ISO E11, strain : BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### Clone Number/Name
**Zm Z-ISO E12**, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag.

**Clone type:** Expression cDNA clone

### Clone Description:

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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### Constructed by:
Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

### Purified by:

**DNA Location** (−20°C): 11, F2

Tube labeled as: Zm Z-ISO E12

**Strain Location** (−80°C): 21, F2

Tube labeled as: Zm Z-ISO E12, strain: BL21 (DE3)

### NOTE:


### Lab Notebook to reference:
Jesús Beltrán

### Original clone name (if different):

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

### Vector size:

**Antibiotic markers:** Ampicillin

### Restriction enzyme(s) to release insert:

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database yes/ No:** 718

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**Clone Description:**

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, F3  
Tube labeled as: Zm Z-ISO F1

**Strain Location** (-80°C): 21, F3  
Tube labeled as: Zm Z-ISO F1, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):  
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

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**Clone Description:**

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available  

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**Fragment size**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C): 11, F4  
Tube labeled as: Zm Z-ISO F2

**Strain Location** (-80°C): 21, F4  
Tube labeled as: Zm Z-ISO F2, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database** yes/ No_720

**Clone Number/Name:** Zm Z-ISO F3, contains Z-ISO (truncation, begins at P75) with a 10X His-MBP-TEV tag.  
**Clone type:** Expression cDNA clone

**Clone Description:**  
**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available  

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**  
**DNA Location** (-20°C): 11, F5  
Tube labeled as: Zm Z-ISO F3  
**Strain Location** (-80°C): 21, F5  
Tube labeled as: Zm Z-ISO F3, strain: BL21 (DE3)

**NOTE:**  
Lab Notebook to reference: Jesús Beltrán  
**Original clone name (if different):**  
**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database #516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### WURTZEL LAB
### CLONE INFORMATION

Date Today: 04/10/2012

Entered into database yes/ No: 721

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#### Clone Description:

**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.

Primers used for amplification from ZmZ-ISO are: information not available

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#### Constructed by:

Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

**DNA Location** (-20°C): 11, F6

Tube labeled as: **Zm Z-ISO F4**

**Strain Location** (-80°C): 21, F6

Tube labeled as: **Zm Z-ISO F4**, strain: BL21 (DE3)

#### NOTE:


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database yes/ No:** 722

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**Clone Description:**
- **Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.
- Primers used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

- **DNA Location** (-20°C); 11, F7
  - Tube labeled as: Zm Z-ISO F5
- **Strain Location** (-80°C) 21, F7
  - Tube labeled as: Zm Z-ISO F5, strain : BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

- Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No 723

**Clone Number/Name:** Zm Z-ISO F6, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag.  
**Clone type:** Expression cDNA clone

**Clone Description:**  
**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.  
Primers used for amplification from ZmZ-ISO are: information not available  
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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**

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**NOTE:**  
Lab Notebook to reference: Jesús Beltrán  
Original clone name (if different):  
Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized : database # 516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database** yes/No: 724

**Clone Number/Name:** Zm Z-ISO F7, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag.  
**Clone type:** Expression cDNA clone

**Clone Description:**  
**Brian:** The vector places a 10X His-MBP-TEV tag for protein purification.  
Primer used for amplification from ZmZ-ISO are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**

**DNA Location** (-20°C): 11, F9 
**Tube labeled as:** Zm Z-ISO F7

**Strain Location** (-80°C): 21, F9 
**Tube labeled as:** Zm Z-ISO F7, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized: database #516

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### WURTZEL LAB
#### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database yes/ No:** 

**Clone Number/Name:** Zm Z-ISO F8, contains Z-ISO (truncation) with a 10X His-MBP-TEV tag.  
**Clone type:** Expression cDNA clone

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**Note:**

- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized: database # 516

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**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
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**Clone Description:**
- **Brian:** The vector places a 10X His-TEV tag for protein purification.
- Primers used for amplification from NnrU are: information not available
  - **Number** 5’ Sequence 3’

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location** (-20°C): 11, G1
- Tube labeled as: NnRU A1
- **Strain Location** (-80°C): 21, G1
- Tube labeled as: NnRU A1, strain: BL21 (DE3)

**NOTE:**
- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- **Organism source of gene:** *Agrobacterium tumefaciens* C58

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
## WURTZEL LAB
### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database** yes/No _727_

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**Clone Description:**

**Brian:** The vector places a 10X His-TEV tag for protein purification.

Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location (-20°C):** 11, G2

Tube labeled as: NnrU A2

**Strain Location (-80°C):** 21, G2

Tube labeled as: NnrU A2, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

**Organism source of gene:** *Agrobacterium tumefaciens* C58

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database** yes/ No  728

| **Clone Number/Name:** NnrU A3, contains NnrU  (full length) with a 10X His-TEV tag. |
|**Clone Type:** Expression cDNA clone |

**Clone Description:**
**Brian:** The vector places a 10X His-TEV tag for protein purification.  
Primers used for amplification from NnrU are: information not available

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**Fragment size**

**Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
**DNA Location** (-20°C) : 11, G3  
Tube labeled as: NnrU A3  
**Strain Location** (-80°C) : 21, G3  
Tube labeled as: NnrU A3, strain : BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** *Caulobacter crescentus*

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

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**Clone Description:**
- **Brian:** The vector places a 10X His-TEV tag for protein purification.
- Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location (-20°C):** 11, G4
- Tube labeled as: NnrU A4
- **Strain Location (-80°C):** 21, G4
- Tube labeled as: NnrU A4, strain: BL21 (DE3)

**NOTE:**
- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- Organism source of gene: *Rhodopseudomonas palustris* CGA009

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
## WURTZEL LAB

**CLONE INFORMATION**

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**Brian:** The vector places a 10X His -TEV tag for protein purification.

Primers used for amplification from NnrU are: information not available

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### Constructed by:

Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

**DNA Location** (-20°C): 11, G5

Tube labeled as: NnrU A5

**Strain Location** (-80°C): 21, G5

Tube labeled as: NnrU A5, strain: BL21 (DE3)

### NOTE:


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: *Rhodopseudomonas palustris* CGA009

### Cloning vector used:

pMCSG7-10x His

### Vector size:

### Antibiotic markers:

Ampicillin

### Restriction enzyme(s) to release insert:

### Sequence verified:

Junction verified:
Clone Number/Name: **NnrU A6**, contains NnrU (full length) with a 10X His-TEV tag.

**Clone type:** Expression cDNA clone

**Clone Description:**
**Brian:** The vector places a 10X His-TEV tag for protein purification.

Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:
**DNA Location** (-20°C): 11, G6
Tube labeled as: **NnrU A6**

**Strain Location** (-80°C): 21, G6
Tube labeled as: **NnrU A6**, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: *Silibacter pomeroyi* DSS-3

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database** yes/ No_732

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**Clone Description:**

**Brian:** The vector places a 10X His -TEV tag for protein purification.

Primers used for amplification from NnrU are: information not available

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**Construct**

**Built by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C) : 11, G7

Tube labeled as: **NnrU A7**

**Strain Location** (-80°C) : 21, G7

Tube labeled as: **NnrU A7**, strain : BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** *Silibacter pomeroyi* DSS-3

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

**Date Today:** 04/10/2012  **Entered into database** yes/ No_733

| Clone Number/Name: | NnrU A8, contains NnrU (full length) with a 10X His-TEV tag. |
| Clone type: | Expression cDNA clone |

**Clone Description:**
**Brian:** The vector places a 10X His-TEV tag for protein purification.
Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location** (-20°C) : 11. G8
Tube labeled as: NnrU A8

**Strain Location** (-80°C) : 21, G8
Tube labeled as: NnrU A8, strain: BL21 (DE3)

**Note:**

**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** *Rhodospirillum rubrum*

**Cloning vector used:** pMCSG7-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
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Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a 10X His-TEV tag for protein purification.
Primers used for amplification from NnrU are: information not available

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constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:
DNA Location (-20°C): 11, G9
Tube labeled as: NnrU A9
Strain Location (-80°C): 21, G9
Tube labeled as: NnrU A9, strain: BL21 (DE3)

NOTE:
Lab Notebook to reference: Jesus Beltran
Original clone name (if different):
Organism source of gene: Ralstonia metallidurans CH34

Cloning vector used: pMCSG7-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

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**Clone type:** Expression cDNA clone

**Clone Description:**  
**Brian:** The vector places a 10X His–MBP-TEV tag for protein purification.  
Primers used for amplification from NnrU are: information not available  
Number 5’ Sequence 3’

**Construct**  
**Built**  
**DNA Location (-20°C):** 11, G10  
**Strain Location (-80°C):** 21, G10  
**Purified by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**NOTE:**  

**Lab Notebook to reference:** Jesús Beltrán  
**Original clone name (if different):**  
**Organism source of gene:** Agrobacterium tumefaciens C58  
**Cloning vector used:** pMCGS9-10x His  
**Vector size:**  
**Antibiotic markers:** Ampicillin  
**Restriction enzyme(s) to release insert:**  
**Sequence verified:** Junction verified:
WURTZEL LAB
CLONE INFORMATION

Date Today: 04/10/2012

Entered into database yes/ No_736

Clone Number/Name: NnrU B2, contains NnrU (full length) with a 10X His-MBP-TEV tag.
Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a 10X His–MBP-TEV tag for protein purification.
Primers used for amplification from NnrU are: information not available

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fragment size

 Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:
DNA Location (-20°C) : 11, H1
Tube labeled as: NnrU B2
Strain Location (-80°C) : 21, H1
Tube labeled as: NnrU B2, strain : BL21 (DE3)

NOTE:


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Agrobacterium tumefaciens C58

Cloning vector used: pMCSG9-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
WURTZEL LAB
CLONE INFORMATION

Date Today: 04/10/2012

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Clone Description:

Brian: The vector places a 10X His –MBP-TEV tag for protein purification.
Primers used for amplification from NnrU are: information not available

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fragment size

Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:

DNA Location (-20°C): 11, H2
Tube labeled as: NnrU B3

Strain Location (-80°C): 21, H2
Tube labeled as: NnrU B3, strain: BL21 (DE3)

NOTE:


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

Organism source of gene: *Caulobacter crescentus*

Cloning vector used: pMCSG9-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database** yes/ No_738

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**Clone Description:**
- **Brian:** The vector places a 10X His–MBP-TEV tag for protein purification.

**Primers used for amplification from NnrU are:** information not available

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**Fragment size**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

- **DNA Location** (-20°C) : 11, H3
  - Tube labeled as: **NnrU B4**
- **Strain Location** (-80°C) 21, H3
  - Tube labeled as: **NnrU B4**, strain : BL21 (DE3)

**NOTE:**

- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- Organism source of gene: *Rhodopseudomonas palustris* CGA009

**Cloning vector used:** pMCSG9-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### Clone Information

**Clone Number/Name:** NnrU B5, contains NnrU (full length) with a 10X His-MBP-TEV tag.  
**Clone type:** Expression cDNA clone

**Clone Description:**  
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Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**

**DNA Location** (-20°C): 11, H4  
Tube labeled as: NnrU B5

**Strain Location** (-80°C): 21, H4  
Tube labeled as: NnrU B5, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** *Rhodopseudomonas palustris* CGA009

**Cloning vector used:** pMCSG9-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**Date Today:** 04/10/2012  
Entered into database **ye...**

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<td>Brian</td>
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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
Purified by:  
**DNA Location** (-20°C): 11, H5  
**Strain Location** (-80°C): 21, H5  
Tube labeled as: **NnrU B6**  
**NnrU B6**, strain: BL21 (DE3)  

**NOTE:**  
Lab Notebook to reference: Jesús Beltrán  
Original clone name (if different):  
Organism source of gene: *Silicibacter pomeroyi* DSS-3  
**Cloning vector used:** pMCSG9-10x His  
**Vector size:**  
**Antibiotic markers:** Ampicillin  
**Restriction enzyme(s) to release insert:**  
**Sequence verified:** Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database:** yes/No_741

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**Clone Description:**

**Brian:** The vector places a 10X His–MBP-TEV tag for protein purification.

Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

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<tr>
<td>Tube labeled as:</td>
<td>NnrU B7</td>
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**Strain Location:** -80°C | 21, H6 |

| Tube labeled as: | NnrU B7, strain: BL21 (DE3) |

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

**Organism source of gene:** *Silicibacter pomeroyi* DSS-3

**Cloning vector used:** pMCSG9-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**Clone Information**

**Date Today:** 04/10/2012  
**Entered into database:** yes/No_742

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**Clone Description:**
- **Brian:** The vector places a 10X His–MBP-TEV tag for protein purification.
- Primers used for amplification from NnrU are: information not available
  - Number
  - 5’ Sequence
  - 3’

**Fragment size**

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location (-20°C):** 11, H7
- Tube labeled as: NnrU B8
- **Strain Location (-80°C):** 21, H7
- Tube labeled as: NnrU B8, strain: BL21 (DE3)

**NOTE:**

- Lab Notebook to reference: Jesús Beltrán
- Original clone name (if different):
- Organism source of gene: *Rhodospirillum rubrum*

**Cloning vector used:** pMCSG9-10x His

**Vector size:**

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified
Clone Number/Name: NnrU B9, contains NnrU (full length) with a 10X His-MBP-TEV tag.
Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a 10X His–MBP-TEV tag for protein purification.
Primers used for amplification from NnrU are: information not available

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constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

Purified by:
DNA Location (-20°C): 11, H8
Tube labeled as: NnrU B9
Strain Location (-80°C): 21, H8
Tube labeled as: NnrU B9, strain: BL21 (DE3)

NOTE:

Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
Organism source of gene: Ralstonia metallidurans CH34

Cloning vector used: pMCSG9-10x His

Vector size:

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

Date Today: 04/10/2012  
Entered into database yes/ No_744

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**Clone Description:**

*Brian:* The vector places a TEV-10x His tag for protein purification.  
Primers used for amplification from NnrU are: information not available  
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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
Purified by:  
**DNA Location (-20°C):** 11, H9  
Tube labeled as: NnrU C1  
**Strain Location (-80°C):** 21, H9  
Tube labeled as: NnrU C1, strain: BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):  
**Organism source of gene:** *Agrobacterium tumefaciens* C58

**Cloning vector used:** pNYCOMPS C-term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
WURTZEL LAB  
CLONE INFORMATION

Date Today: 04/10/2012  
Entered into database yes/ No_745

Clone Number/Name: NnrU C2, contains NnrU (full length) with a TEV-10x His.
Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a TEV-10x His tag for protein purification.
Primers used for amplification from NnrU are: information not available

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fragment size

Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:
DNA Location (-20°C): 11, H10
Tube labeled as: NnrU C2
Strain Location (-80°C): 21, H10
Tube labeled as: NnrU C2, strain: BL21 (DE3)

NOTE:


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Agrobacterium tumefaciens C58

Cloning vector used: pNYCOMPS C-term.

Vector size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
**WURTZEL LAB**  
**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No_746

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**Clone Description:**
- **Brian:** The vector places a TEV-10x His tag for protein purification.  
- Primers used for amplification from NnrU are: information not available
  - **Number** 5’ **Sequence** 3’

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**
- **DNA Location** (-20°C): 11, I1
  - Tube labeled as: NnrU C3
- **Strain Location** (-80°C): 21, I1
  - Tube labeled as: NnrU C3, strain: BL21 (DE3)

**NOTE:**

- Lab Notebook to reference: Jesús Beltrán
- **Original clone name (if different):**
- **Organism source of gene:** *Caulobacter crescentus*
- **Cloning vector used:** pNYCOMPS C-term.
- **Vector size:**
- **Antibiotic markers:** Kanamycin
- **Restriction enzyme(s) to release insert:**
- **Sequence verified:** Junction verified:
## Clone Information

### Date Today: 04/10/2012

**Clone Number/Name**: NnrU C4, contains NnrU (full length) with a TEV-10x His.

**Clone type**: Expression cDNA clone

### Clone Description:

**Brian**: The vector places a TEV-10x His tag for protein purification.

Primers used for amplification from NnrU are: information not available

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### Constructed by:

Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by**: DNA Location (-20°C) : NnrU C4

Tube labeled as: 11, I2

**Strain Location** (-80°C) : NnrU C4

Tube labeled as: 21, I2, strain : BL21 (DE3)

### Note:


- Lab Notebook to reference: Jesús Beltrán

- Original clone name (if different):

**Organism source of gene**: *Rhodopseudomonas palustris* CGA009

**Cloning vector used**: pNYCOMPS C-term.

**Vector size**: 

**Antibiotic markers**: Kanamycin

**Restriction enzyme(s) to release insert**: 

**Sequence verified**: Junction verified:
**WURTZEL LAB**

**CLONE INFORMATION**

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**Clone type:** Expression cDNA clone

**Clone Description:**

- **Brian:** The vector places a TEV-10x His tag for protein purification.
- Primers used for amplification from NnrU are: information not available

**Number** | **5’ Sequence** | **3’**
---|---|---

**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

- **DNA Location** (-20°C): 11, I3
- Tube labeled as: NnrU C5
- **Strain Location** (-80°C): 21, I3
- Tube labeled as: NnrU C5, strain: BL21 (DE3)

**NOTE:**


**Lab Notebook to reference:** Jesús Beltrán

**Original clone name (if different):**

**Organism source of gene:** *Rhodopseudomonas palustris* CGA009

**Cloning vector used:** pNYCOMPS C-term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
### WURTZEL LAB
#### CLONE INFORMATION

**Date Today:** 04/10/2012  
**Entered into database:** yes/ No_749

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#### Clone Description:
- **Brian:** The vector places a TEV-10x His tag for protein purification.
- Primers used for amplification from NnrU are: information not available

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**Construct by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027

**Purified by:**

**DNA Location (-20°C):** 11, I4  
**Tube labeled as:** NnrU C6

**Strain Location (-80°C):** 21, I4  
**Tube labeled as:** NnrU C6, strain : BL21 (DE3)

**NOTE:**

- **Lab Notebook to reference:** Jesús Beltrán
- **Original clone name (if different):**

**Organism source of gene:** Silicibacter pomeroyi DSS-3

**Cloning vector used:** pNYCOMPS C-term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
Clone Number/Name: NnrU C7, contains NnrU (full length) with a TEV-10x His.
Clone type: Expression cDNA clone

Clone Description:
Brian: The vector places a TEV-10x His tag for protein purification.
Primers used for amplification from NnrU are: information not available

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Constructed by: Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
Purified by:
DNA Location (-20°C) : 11, I5
Tube labeled as: NnrU C7
Strain Location (-80°C) 21, I5
Tube labeled as: NnrU C7, strain: BL21 (DE3)

NOTE:
Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):
Organism source of gene: Silicibacter pomeroyi DSS-3

Cloning vector used: pNYCOMPS C-term.
Vector size:
Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert:

Sequence verified: Junction verified:
### WURTZEL LAB

**CLONE INFORMATION**

**Date Today:** 04/10/2012  
**Entered into database yes/ No:** Yes

| Clone Number/Name: NnrU C8, contains NnrU (full length) with a TEV-10x His.  
| **Clone type:** Expression cDNA clone |

**Clone Description:**

**Brian:** The vector places a TEV-10x His tag for protein purification.  
Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027  
**Purified by:**

**DNA Location (-20°C):** 11, I6  
**Tube labeled as:** NnrU C8

**Strain Location (-80°C):** 21, I6  
**Tube labeled as:** NnrU C8, strain : BL21 (DE3)

**NOTE:**


Lab Notebook to reference: Jesús Beltrán

Original clone name (if different):

**Organism source of gene:** *Rhodospirillum rubrum.*

**Cloning vector used:** pNYCOMPS C-term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
**WURTZEL LAB**
**CLONE INFORMATION**

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**Clone Description:**
**Brian:** The vector places a TEV-10x His tag for protein purification.
Primers used for amplification from NnrU are: information not available

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**Constructed by:** Brian Kloss, Ph.D, Senior Research Associate, NYCOMPS NYSBC Park Building 89 Convent Avenue New York, NY 10027
**Purified by:**
**DNA Location** (-20°C): 11, I7
Tube labeled as: NnrU C9
**Strain Location** (-80°C): 21, I7
Tube labeled as: NnrU C9, strain: BL21 (DE3)

**NOTE:**
**Lab Notebook to reference:** Jesús Beltrán
**Original clone name (if different):**

**Organism source of gene:** *Ralstonia metallidurans* CH34

**Cloning vector used:** pNYCOMPS C-term.

**Vector size:**

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:**

**Sequence verified:** Junction verified:
WURTZEL LAB
CLONE INFORMATION

Date Today: 7/12/2010 Entered into database yes/ No_579

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<td>Clone type: Expression cDNA clone</td>
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**Clone Description:**

ZmZ-ISO1.1 (Data sheet # 497), CDS from maize B73 (without transit sequence, first 46aa). The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein.

Restriction sites: *BamHI* and *SalI*

Primers used for amplification from ZmZ-ISO1 are

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<td>GC GTCGAC CTACCAGGAAGTTGGTAGCT</td>
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Fragment size: 984bp

**Construct by:** Jesú s Beltrán, 2010

**Purify by:** Jesú s Beltrán

DNA Location (-20°C) Box Number # 10 Position: D3 Conc. 20ng/ul

Tube labeled as: pCol Zm-ZISO 135 H/A

Strain Location (-80°C) 19, H8 (Bl21DE3) 19, H10 (C43 DE3)

Tube labeled as: BL21 (DE3) Zm ISO NTP, C43 (DE3) Zm ISO NTP

**NOTE:** This truncated version is functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesú s Beltrán

Original clone name (if different):

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1

Vector size: 3719bp Insert size:

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI
| GATATACCATTGGGAGACGACCCATACCATACATCACCACAGCCAGGATCCTCACCAGC   |
| TCGTCCCCGCGTGCAGGCGGAGGAGACATCGAGCCAGCCAGGAGGAGGCGTTGTTGCG   |
| GAGGGGAGCCAGAGTCTGGGCGAGGGCCCTGCTGCTGGGAGGATTCGGCCTGCTGCT   |
| TCGAGCTCAAGGACCCAGACGTGGGACGTGTTGACCTCGCCCCAGGATACTAGG   |
| TCGGCTGCTCGGTGCGCTCAGTGCTGTGGATCGACCACAGTGACCGGGTCGGG   |
| ACCAAATTCCCTCGACGTGCTGCGCTCCGCTCCGCTCCGACAGCCACAGGAGGTTATGTT   |
| GCTCCTTACCATAATTTTTGGCTGTAGTTCCATAGTGGATAGGCAAGGCTACCGGAAA   |
| GTGGTGAGAAAAATAGTGAGGGAGCTGTGTTTACCCGCTGTGCTGTCTGGAGATTTC   |
| ACTGCTTTTACGAGTTACTACTATTGATACTTTCAAAATATCAGGGGAAACAGGAATAATGCGATATGTA   |
| CTCAATTATGGCAAGGTTCCAGGGAAATCATGCCATTCATGAGCTTTCTTGTGCTC   |
| TCTGTTTCTGTTCTCTCTTCTGTATCCATCCACTTTCAATCTCTTTGGAAGTGGCA   |
| GCTGTTGAGAAAATAGTGAGGGAGCTGTGTTTACCCGCTGTGCTGTCTGGAGATTTC   |
| ACTGCTTTTACGAGTTACTACTATTGATACTTTCAAAATATCAGGGGAAACAGGAATAATGCGATATGTA   |
| CTCAATTATGGCAAGGTTCCAGGGAAATCATGCCATTCATGAGCTTTCTTGTGCTC   |
| TCTGTTTCTGTTCTCTCTTCTGTATCCATCCACTTTCAATCTCTTTGGAAGTGGCA   |
| GCTGTTGAGAAAATAGTGAGGGAGCTGTGTTTACCCGCTGTGCTGTCTGGAGATTTC   |
| ACTGCTTTTACGAGTTACTACTATTGATACTTTCAAAATATCAGGGGAAACAGGAATAATGCGATATGTA   |
| CTCAATTATGGCAAGGTTCCAGGGAAATCATGCCATTCATGAGCTTTCTTGTGCTC   |
| TCTGTTTCTGTTCTCTCTTCTGTATCCATCCACTTTCAATCTCTTTGGAAGTGGCA   |
| GCTGTTGAGAAAATAGTGAGGGAGCTGTGTTTACCCGCTGTGCTGTCTGGAGATTTC   |
| ACTGCTTTTACGAGTTACTACTATTGATACTTTCAAAATATCAGGGGAAACAGGAATAATGCGATATGTA   |

**NOTES:** Conditions for expression are essential as reported by Chen et al., 2010.
Underline seq.: His tag
Clone Number/Name: pCol Zm Z-ISO NTP Y340A
Clone type: Expression cDNA clone

Clone Description:
ZmZ-ISO1.1 (Data sheet # 579), CDS from maize B73 (without transit sequence) was mutated in Y340 by A. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from ZmZ-ISO1 are

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<td>3028</td>
<td>cgggatctcaagctgtcccgcggcgcg</td>
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<td>2587</td>
<td>GC GTCGAC CTACCAGGGAAGTTGGTAGCT</td>
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Constructed by: Jesús Beltrán, 2010
Purified by: Jesús Beltrán

DNA Location (-20°C) Box Number # 12 Position: A8 Conc. 20ng/ul
Tube labeled as: pCol Zm-ZISO 340 Y/A

Strain Location (-80°C)
XL10 Gold : Box Number: 22 Position: C3. tube labeled as: pCol Zm Z-ISO 340 Y/A EBP : Box Number: 22 Position: C4. tube labeled as: EBP pCol ZmZ-ISO 340 Y/A

NOTE:

NOTE: This truncated version is functional as demonstrated by complementation in E. coli


Lab Notebook to reference: Jesús Beltrán
Original clone name (if different):

Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1  
Vector size: 3719bp  
Insert size:

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<tr>
<th>Antibiotic markers:</th>
<th>Kanamycin</th>
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</table>

| Restriction enzyme(s) to release insert:  | BamHI and SalI |

| Sequence verified:  | Yes  
| Junction verified:  | |

GATTTCCCTGTAGAAATAATTTTGTAAAATAAAGAGATATACACCATTGGGGCAGCAGCCCATCACCATCATACACCACAGCCAGGATTCCTCAGCTGTCGTCGCCGCCCCGTGCCTGCGGGGAGGACATCGAGCCGAAGGAGGGCAGTTGGCGGGAGGGCCGTGCTGGGGAGGGATTCGGCTGCATTACGCTCAAGGACAGAGCGTGGCGTCGGGCTTACTTCGCCGGGATAATCTACGTGAGGACAAGAAATTCCTGATTTTGTGCTGTCACATAGTGATGGCAGCTAACGCTACCCGACAGGATACGCTAACGTGCCGAAAAAGTGTTGAGAAAATAGTGGGGAGCCGTGGTTTACCGTGCTGTTCCGTGTCGTGAAATTCCTACGTGCTGCTTAAGCAGTTACATATTGATAACTACCATAATCATCGGTAGTTGATGATGCTACTAGTATGAGGCTCTACATATAGTGGGGGAGCGTGTTTACCGTGTGCTGTTCGCTGGAATTTCACTGCCTTTAGCAGTTACTACTATTGATAACTACCTGACAATATTGAGGCTCTTTGGTTCTCGTTCATTTCGTTCTTCTTCTTTCTGATCCTACCACTCTATCAATCTCTTGGAAGTGCGACGCTGACGAAGACGCCTAAAATTACACATGTGGGAAAAACAGGAAATAATGCGTATCACCAAGCATCCACAGGGATGTTGGTCGGAATTTGGTGTCATTACACGAGGCTGTTGTCACGCTATGGTGAAGCCTTCGAAGTACTGAAGAAGAGAACAAGTGTTATGCCCTTCGCTGCGATCATCGATGGACGGCAGAAACTGCCCAAGATTATCACAAGGAGTTTTGGTTACCAGCTGTAGCAATCACAATGTTAACCTTGCGGTGCATACTTTGCTCATCCATTGATGCAAGCATCCAGCTACCAACTTCCCTGGTAGGTCGACAAG

NOTES: Conditions for expression are essential as reported by Chen et al., 2010.  
Underline seq.: His tag
WURTZEL LAB
CLONE INFORMATION
Depositor’s Name: Charles Ampomah-Dwamena

Date Today: 6/17/2011 Entered into database yes/ No_619

| Clone Number/Name: MCSG9 Z-ISO E2 H150/A |
| Clone type: Expression cDNA clone |

**Clone Description:**
The vector places a 10X His tag, in addition to MBP, on the N-terminus Clone E2 (aka 79.1) is two amino acids shorter and begins with the Arginine at AA position 49. Clone was created by site-directed mutagenesis of MCSG9 Z-ISO E2 (datasheet # 582) residue H150 to A Primers used for site-directed mutagenesis of E2

**Number** | **Label** | **Sequence**
--- | --- | ---
3140 | H150/A sense | TATTTTTGCGGTGGTGGCTAGCGGCATGGCGAG
3141 | H150/A antisense | GCTCGCCATGCCGCTAGCCACCACCCGCAAAAAATA

**Constructed by:** Charles Ampomah-Dwamena

**Purified by:**

**DNA Location** (-20°C): Box 10 I8, Conc.: 40ng/ul; Tube labeled as: Z-ISO E2 H150/A

**Strain location** (-80°C) Box 20 C9 & C10; Tube labeled as: MCSG9 Z-ISO E2 H150/A

XL10  Box: 22 D 9 Tube labeled as: MPB Z-ISO E2 H150/A,C43 (DE3)

Lab Notebook to reference: Charles Ampomah-Dwamena Book # 43649

Original clone name (if different):

Organism source of gene: A synthetic Z-ISO gene, ACA less and codon optimized


Vector size: 6.4 kb

Antibiotic markers: Ampicillin

Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: YES Junction verified: YES

GATTTAAAAAACAACATGAATGCAGACACCGATTACTCCATCGCAGAAGCT
GCCCTTAATAAAAGCGGAACAGCGATGACCATCAACCGCGCCGGTCAGTG
CCAACATCGACACCACGAAAATGTAATTATGGTGTACCGTACTGCCGACCCTT
CAAGGGTCAACCATCCAAAACCGTTCTGGCCGCTGACTGCCAGGTATTAAC
GCCGCCAGTCCGAACAAAGAGCTGGGCAAAAAGAGTCTCTCGAAAAACTATCTGCC
TAGCTGATGAAGGTTGGAACGCTTAAATAAAGACAAACCCGCTGGGATGCCGT
AGCGCTGAGGCTTAAAGAGGCTGAAATGCTACGGCCTACGCTCGCCGAGCTG
ACCATGAAACGCGCAGAAAGTGAAAATCATGGCAGACCTCCGCAGATGTT
CCGCTTTCTGTTATGCGCGCTGCTACTGCGGTGATCAACGGCCAGCGGTCC
TCAGACTGTGCTGAAGCTGCCCTGAAGACGCCAGACTGATTACGATATTTCA
GGTACCGAAGCCTGAGCTGGGCACTGAGGCATCTTATCTGGGGCAGACCTG
TGACTGATGAAGGGTGTAATGCAGCAGCTCTGGGTGCTTCTGGGTGAGCCTG
GCCGCCGCGCCCGGCTTCTGGTATGCACTGCGCTGATCAACGGCCAGCGGTCC
TCAGACTGTGCTGAAGCTGCCCTGAAGACGCCAGACTGATTACGATATTTCA
GGTACCGAAGCCTGAGCTGGGCACTGAGGCATCTTATCTGGGGCAGACCTG
WURTZEL LAB
CLONE INFORMATION
Depositor’s Name: Charles Ampomah-Dwamena

Date Today:  6/17/2011
Entered into database yes/ No_620

| Clone Number/Name: | MCSG9 Z-ISO E2 H266/A |
| Clone type: | Expression cDNA clone |

**Clone Description:**
The vector places a 10X His tag, in addition to MBP, on the N-terminus Clone E2 (aka 79.1) is two amino acids shorter and begins with the Arginine at AA position 49. Clone was created by site-directed mutagenesis of MCSG9 Z-ISO E2 (datasheet # 582) residue H266 to A

Primers used for site-directed mutagenesis of E2

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<tr>
<td>3142</td>
<td>H266/A sense</td>
<td>GATTTGGTGCCCTGGCGGCTACCCTGGATTGGC</td>
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<tr>
<td>3143</td>
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<td>GCCAATCCACAGGGTAGCCGCCAGGCACCAAATC</td>
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**Constructed by:** Charles Ampomah-Dwamena,
**Purified by:**
**DNA Location** (-20°C): Box 10 I9, Conc.: 40ng/ul; **Tube labeled as:** Z-ISO E2 H266/A
**Strain location** (-80°C): Box 20 D1 & D2; **Tube labeled as:** MCSG9 Z-ISO E2 H266/A
**TOP10 Box:** 22  D 10 **Tube labeled as:** MPB Z-ISO E2 H266/A_C43 (DE3)

Lab Notebook to reference: Charles Ampomah-Dwamena Book # 43649

**Original clone name (if different):**

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized


**Vector size:** 6.4 kb

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:** BamHI and Sall

**Sequence verified:** YES Junction verified: YES

**Sequence:**

GCAAGAACCCTACTTACACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTTATGAAGAAGGCAAGTGACTACATTTAAAGACGGTGCGGCTGGGATACGGCCTGGCAGACCGGCTTCTGACCTTCTGTTGACCTGATTTAATAAGCAAACAGACTGCAACACCAGATTTACTCCATCGACAGAAGCTGCCTTTAATAAAGGCAGAACAGCGATGACCACATACCGGCGTGGGCACTGGTCCAACATCGACACCA
Date Today: 6/17/2011

Entered into database yes/ No: 801

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<th>Clone Number/Name:</th>
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<td>Clone type; Expression cDNA clone</td>
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**Clone Description:**
The vector places a 10X His tag, in addition to MBP, on the N-terminus Clone E2 (aka 79.1) is two amino acids shorter and begins with the Arginine at AA position 49. Clone was created by site-directed mutagenesis of MCSG9 Z-ISO E2 (datasheet # 582) residue H150 to A

Primers used for site-directed mutagenesis of E2

<table>
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<th>Number</th>
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<tr>
<td>3140</td>
<td>C263/A sense</td>
<td>GGCCAGGTTGATTGGGCTGGGCATACCC</td>
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<td>3141</td>
<td>C263/A antisense</td>
<td>GGGTATGCGCCAGGGCCAAATCACCTG GCC</td>
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**Constructed by:** Jesus Beltran

**Purified by:**

**DNA Location** (-20°C): Box 12 C1, Conc.: 100ng/ul; **Tube labeled as:** MBP Z-ISO E2 C263A

**Strain location** (-80°C) Box 22 F2; Tube labeled as: MBP Z-ISO E2 XL10 Box: 22 F 1 Tube labeled as: MPB Z-ISO E2 C263/A_C43 (DE3)

Lab Notebook to reference: Jesus Beltran

Original clone name (if different):

**Organism source of gene:** A synthetic Z-ISO gene, ACA less and codon optimized


Vector size: 6.4 kb

**Antibiotic markers:** Ampicillin

**Restriction enzyme(s) to release insert:** BamHI and SalI

Sequence verified: YES Junction verified: YES

```
GACCGGAGCTCGAATTTCGGATCCGGTTATCCACTTCAATCTTACCACGGCGACGTGAT
AGCTGCTCCTGCATCAGCGGCAGGGATGCGCAAAATACCGCGCCAGGGTCAGCATGGT
AATCGGCCACATACCGGCAGACGAAATAATTCTTTATGATAATCTTCTTCGCGCAGTTTCT
GACCGCCATCAAATACGCGCCAACACGGCGATCAGCCTGTGGTGTCAGCTTATCAGCAC
```
TTCAAAACGCTTTCCGCCCTAACGCTTCAGCAGACGACGATCGCCGTTCCACGCAGCCA
AACAGATGATGGCTAATCAGGCCACGCCTCGCCGCCACCGCCACCGCCACGCTATTGCCAA
TCCACAGGGTATGCGCCAGGGCCAGAAAATCACCTGGCCACCACACCATCTCTGCAGGATGACG
GGTAATACGATTAATGCCGGTTTCCACCACATATGCAGTTTCGTTTATCCACCGCCCG
CCACTTCCAGCAAGTAAAGGTCGCTCGGATACAGAAAAAGCTAAATAAGCT
GCTAAACCACAGCAGTTTCATGAATGGCGTGATGATGCCCTGCACCTGCACAGCTGG
GTGCCATCATAACGATGGTTAAATAAATACACATGTTGTCACCGCCAGCGGCA
GGCTAATGCAGGGCAAAACAGCAGATACACACGTTGGCCCAAAATTTTTTTCGCC
GCTTTACGGCAGGCTCAGCCATGCGCTATGCAACCACCAGCAGAATAATTGTCAGC
AGCAGCATCACCACCCTTCATGGCTATCGCTACGCTCGCCACCGCATCCAGAAATTT
GGTGCCACACGCCGTGCTCGGATCAATCCACAGCAGTTCAGCGCCACCAGCACC
GGCAGAATGCCCAGCAAAATACGCCACGCTCGCCACGCTCTGATCTTTCAGTT
CAAAACGCCCGCTATCTCTCAGCCCACCAGCACCAGGGCCGCGGAGGTTCATTCGCCT
TCCGCCACACGCCCTTTTCTGTTTCAATGCCGCCGCGCCACCG
WURTZEL LAB
CLONE INFORMATION
Depositor’s Name: Charles Ampomah-Dwamena

Date Today: 6/17/2011 Entered into database yes/ No_610

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<th>Clone Number/Name: &gt;pCOL ZmZISO.77_367 (ZISO T1)</th>
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<tbody>
<tr>
<td>Clone type; Expression cDNA clone</td>
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**Clone Description:**
ZmZISO.77_367 is amplified from pCOI ZmZISO NTP (data sheet # 579) to remove the first 77 aa from the protein. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

Primers used for amplification from pCOL ZmZISO.77_367 are

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<td>3097</td>
<td>CGGGATCCTCTCGTGGGTGAGGATTCGGCTGCGTTC</td>
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PCR fragment size

- 3097
- CGGGATCCTCTCGTGGGTGAGGATTCGGCTGCGTTC 890bp

**Constructed by:** Charles Ampomah-Dwamena

**Purified by:** Charles Ampomah-Dwamena

DNA Location (-20°C) Box 10 H9 Conc: 40 ng/ul; Tube labeled as: pCOLA ZISO. T1

Strain location (-80°C): Box 20 A1, A2; Tube labeled as: pCOLA ZISO T1 BL21

Transformed into: pACCRT-EBP E coli; Strain location: Box 20 B1 & B2; Tube labeled as: EBP ZISO T1

NOTE: This truncated version is functional as demonstrated by complementation in E. coli

Lab Notebook to refer: Charles Ampomah-Dwamena

Original clone name (if different):

Organism source of gene: Maize B73

Cloning vector used: pColaDuet-1

Antibiotic markers: Kanamycin

Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Yes Junction verified: Yes

```
CCATGGCGAGCAAGCCATCCATCACACCACAGCCAGGTACCTCTCTCGGTTGCTAG
GGATTCGGCTGCTCGAGCTCAAGGACCAGACAGGCTGCGTTCGGCTGGCCTACTCTTC
GCCGGGATATGAGGTGCTGGTACGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
GTACCGGGGTCGGGAGCAAGGATATGCGAGCTCGAGCTCGAGCTCGAGCTCGAGCTCG
AGGTGTTATGTGTGCTCTCTTAGCATAATTTTTGTGTAAGTCATATGTTGTATGG
CAAGCCTACGGGAAATGAGGTGAGAAATAGTGGGGAGGGAGGGTGGTCTACCGTGTCG
GTTGCTGGAATTTCACTGCTTTAGCAGTTACTACTATTGTATATTTCATAATC
```
ATCGGTATGATGGTACTCAATTATGGCAAGTTCTAGGGAATCACTGGCATTCATGAGCTTTTTGGTTCTCGTGCATTCTTCTCCATTCTTTTCTGTATCCATCCACTTTTCAATCTCTTGGAAAGTGGCAGCTGTTGGCAAGCCTAAATTACATGTTGGAAAACAGGATTATGCGTATCACCAGACATCCACAGATGTGGTTGTCAGGTAATTGTTGTCCTTGCCCATACACTATGGATTGGCAACTCAGTTGCCCCGCTAGCGCCCTCTGTCGGACTTATCTAGCCACCATCTCTTTGGTGCTTGGGAATGGTTGACAGGAGGCTGTGTTGTCACGCTATAGTGAAAGCCCTTGGAAAGTACTGAAAGAGAAAGGTCGTTCTTGACTTGCCCAAGGTTGACAGGAGGCTGTGTTGTCACGCTATAGTGAAAGCCCTTGGAAAGTACTGAAAGAGAA

Note: Histag sequence underlined; start and stop codons in bold
**WURTZEL LAB**

**CLONE INFORMATION**

Depositor’s Name: Charles Ampomah-Dwamena

Date Today: 6/17/2011

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| Clone Number/Name: | >pCOL ZmZISO.136_367 (ZISO T2) |
| Clone type: | Expression cDNA clone |

**Clone Description:**

ZmZISO.136_367 is amplified from pCOl ZmZISO NTP (data sheet # 579) to remove the first 136 aa from the protein. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: *BamHI* and *SalI*

Primers used for amplification from pCOL ZmZISO.136_367 are

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<td>713bp</td>
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<td>2587</td>
<td>GCGTCGACCTACCAGGGGAAGTTGGTAGCT</td>
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**Constructed by:** Charles Ampomah-Dwamena

**Purified by:** Charles Ampomah-Dwamena

**DNA Location** (-20°C) Box 10 H10 -- Conc. 40ng/ul; **Tube labeled as:** pCOLA ZISO. T2

**Strain location** (-80°C): Box 20 A3 and A4; **Tube labeled as:** pCOLA ZISO T2 BL21

**Transformed into:** pACCRT-EBP E coli; **Strain location** Box 20 B3 & B4; **Tube labeled as:** EBP ZISO T2

NOTE: This truncated version is non-functional as demonstrated by complementation in E. coli

Lab Notebook to reference: Charles Ampomah-Dwamena

Original clone name (if different):

**Organism source of gene:** Maize B73

**Cloning vector used:** pColaDuet-1

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:** BamHI and SalI

**Sequence verified:** Yes Junction verified: Yes

```
CCATGGGCAGGCAGCCATCACCATCATCACCACAGCCAGGATCTCTGAGGTTGTTAT
GTTGCTCTTTACCATATAATTTTGGCTGTAGTCCATAGTGTTATGGCAAGCTACGGGA
AAAGTGGTGAAATATGGGGGAAGCGTGTACCTACGGTTGCTGCTGGTGGTGGAT
TTACTGCCTTTAGCTACTACTATTGATAGTTAGATCTCAAAAATCATCGGTATGGATG
GTACTCAATTATGGCAAGTCGACAGGAAATACGAGCATTGGGCTGCTGCTGCTGCTG
TCGTCGCTTTCTCTTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
GCAGCTGTGGGACAAGGCTAAAAATTACACATAGTGTTGGAACAGGAATAATGCGTATCA
```
CCAGACATCCACAGATGGTTGTCAGGTAATTGGTGCCCTTGCCCATACACTATGG
ATTGGCAACTCAGTTGCCGCTAGCGGCTCTCTGTCGGACTTATCAGGCCACCATCTCTT
TGGTGCTTGGAATGGTGACAGGAGGCTGTGTCACGCTATGGTGAGAGCCTTCGAA
GTACTGAAGAGAGAACAAAGTGTTATGCCCCTTCGCTGCATCGATCGATGGAGCGGC
AGAAAACGTGCCAAAGATTATCAACAAGGAGTTCTTTCGTTACCATATGTAGCAAT
CACAATGTTAACCTTGGGTGCATACTTTGCTCATCCATTGATGAAGCATECCAGCT
ACCAACTTCCCTGGTAGGTCGACAAGCCTTGCAGGCGCATATAA
Note: Histag sequence underlined; start and stop codons in bold
WURTZEL LAB
CLONE INFORMATION
Depositor’s Name: Charles Ampomah-Dwamena

Date Today: 6/17/2011 Entered into database yes/ No_612

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<th>Clone Number/Name:</th>
<th>&gt;pCOL ZmZISO.47_359 (ZISO T3)</th>
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<tr>
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<td>Expression cDNA clone</td>
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<tr>
<td>Description</td>
<td>ZmZISO.47_359 is amplified from pCol ZmZISO NTP (data sheet # 579) to remove the first 47 aa from the N-terminal and the last 8 aa from the protein. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI</td>
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Primers used for amplification from pCOL ZmZISO.47_359 are

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<td>3099</td>
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<td>594bp</td>
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Constructed by: Charles Ampomah-Dwamena
Purified by: Charles Ampomah-Dwamena

DNA Location (-20°C) Box 10 I1, Conc.: 40ng/ul; Tube labeled as: pCOLA ZISO. T3
Strain location (-80°C): Box 20 A5 & A6; Tube labeled as: pCOLA ZISO T3 BL21
Transformed into: pACCRT-EBP E coli; Strain location: Box 20 B5 & B6; Tube labeled as: EBP ZISO T3

NOTE: This truncated version is non-functional as demonstrated by complementation in E. coli

Lab Notebook to reference: Charles Ampomah-Dwamena
Original clone name (if different):

Organism source of gene: Maize B73
Cloning vector used: pColaDuet-1
Antibiotic markers: Kanamycin
Restriction enzyme(s) to release insert: BamHI and SalI

Sequence verified: Yes Junction verified: Yes

CCATGGCAGCAGCCATCCATCATCATCAACACAGCCAGGTGTCTCCACGCTCGTCC
CCGCCGTGCGGTGGGGAGGACATCGAGCAAGGAGGCATGGTCTTGCACGAGG
AGACGAGTCTGGCAGGAGGGCTGCTCGGTGAGGATTCGCTGCTGCAAGG
CTCAAGGACCAGAGCGTTGCGTGCTGGGTCTTACTTGCCCGGATTAGGTGCGG
TGCTCGTTGCGCTCAACGTGCTGTGGATCGACCCAGTACCGGGGGTCGGGACCAA
ATTCCCTCAGCAGCTGCTGCTGCTGACAGCAGGAGGGTTGTTATGTTGCTC
TTACCATAATTTTTGCTGTAGTCCATAGTTGATGGCAAGCCTACGGGAAGTGGT
GAGAAAATAGGGGGAGCCTGTTTACCCTGCTGGCTTCTCCTGGGAATTTCACTGCCTTTAGCAGTTACTACTATTTGTAATCTTTCAATAATCTCGGTATGATGGTACTCAA
TTATGGCAAGTTCAGGGAATCATTGGCATTCATGAGATTCTTTTGCTTTTCTGCCTGTTGATTTGCTTTTCTTCTGTTATCCATCCACTTTCAATCTCTTGGGAAGTGGCAGCTGTGGACAAGCCTAAAAATTACACATGTGGGAAAACAGGAATAATGCGTATTCACCAAGCATCCACAGATGGTTGTGAGTGAATTCTGCTTCTGGGAACAGGATACGAGGCTTGTTGCTCGGTGCTGCATCGGATCATGGCGGCAAAACTG
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GAAGAGAAAAGTGTTATGCCCTTCCCTGCCGATCGATGGACGGCAGAAACTG
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Note: Histag sequence underlined; start and stop codons in bold
WURTZEL LAB
CLONE INFORMATION
Depositor’s Name: Charles Ampomah-Dwamena

Date Today: 6/17/2011 Entered into database yes/ No_613

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**Clone Description:**
ZmZISO.77_359 is amplified from pCOL ZmZISO NTP (data sheet # 579) to remove the first 77 aa from the N-terminal and the last 8 aa from the protein. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

```
CGGGATCCTCTCTGCGCCTGGGATTCGCTGCGTTC
```

Primers used for amplification from pCOL ZmZISO.77_359 are

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**Constructed by:** Charles Ampomah-Dwamena

**Purified by:** Charles Ampomah-Dwamena

**DNA Location** (-20°C) Box 10 I2, Conc.: 40ng/ul; **Tube labeled as:** pCOLA ZISO. T4

**Strain location** (-80°C): Box 20 A7 & A8; **Tube labeled as:** pCOLA ZISO T4 BL21

**Transformed into:** pACCRT-EBP E coli; **Strain location:** Box 20 B7 & B8; **Tube labeled as:** EBP ZISO T4

**NOTE:** This truncated version is non-functional as demonstrated by complementation in E. coli

**Lab Notebook to reference:** Charles Ampomah-Dwamena

**Original clone name (if different):**

**Organism source of gene:** Maize B73

**Cloning vector used:** pColaDuet-1

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:** BamHI and SalI

**Sequence verified:** Yes Junction verified: Yes

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CCATGGGCGAGCAAGCAATCATCAATCATACAGCCACAGGATCCTCTCTCGTGGTGA
GGATTTCGCGCGCTCAGCTCAAGAGCAGACGCTGGCGCCTCGTGGCTACTTTC
GCCGGGATACATAGGTCCGGTTCTCGTCCCGCTCAACGTGTGTGGATCGACCCCA
GTACCCGGGGTCCGGAAGCAATTTCACTCGACCTGTCGCTCGCTCCCTCGACAGCCA
CGAGGTTTGTGTTGTTGCTTCTCCTATCAATTTTTCGCTGTGAATCTACATGTGATGG
CAAGCCTACGGAAGAGTGGTGAGGAATAATGATGTCGGGGAGAGCTGTGTTTACACGTGTGCT
GTTTCGCTGGAATTTCACTCGCCCTTTAGCAGTTAATCTAATTGTATATATCTACTAAATC
ATCGGTATGATGGTACTCAATTATGGCATGGAATCAGGATTGCTCCTGTCGTCGATCCA
```
GCTTCTTTGGTTCTCGTCGTCATTTTCGTTCTTTCTGTATCCATCCACTTTTCAATCTCTTGGAAGTGGCAGCTGTGGACAAACAGCTAACATGTGGGAAACAGGAAATAATCGGTATCACCAGACATCCACAGATGGTTGGTCAGGTAATTTTGGTGCCCTTTGCCCATACACTATGGATTTGGCACAACCTCAGTTGCCGTAAGCGGCTCCTGTCGGAACATTTACAGCCACCATCTCCTTTGTTGCTTGGGAATGGTGACAGGAGGCTTGTTGTCACGCTATGGTGAAAGCCTTGGAAGTACTGAAGAAGAGAACAAGTGTTATGCCCTTCGCTGCCGATCATCGATGGGACGCGCAGAAACTGCCCAAAAGATTATCACAAGGAGTTCTTTCTGGTTACCATATGTAGCAATCACAATGTTAACCTTTGGGTGCATACTTTGCTCATCCATTGATGCAAGCAGGTGACACAAGCTTGCGGCCGCATATAANote: Histag sequence underlined; start and stop codon in bold
**Clone Number/Name:** >pCOL ZmZISO.136_359 (ZISO T5)

**Clone type:** Expression cDNA clone

**Clone Description:**
ZmZISO.136_359 is amplified from pCOl ZmZISO NTP (data sheet # 579) to remove the first 136 aa from the N-terminal and the last 8 aa from the protein. The gene is cloned in the vector pColaDuet-1 and formed HIS Tag-Z-ISO fusion protein. Restriction sites: BamHI and SalI

**Primers used for amplification from pCOL ZmZISO.136_359 are**

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</tbody>
</table>

**Constructed by:** Charles Ampomah-Dwamena

**Purified by:** Charles Ampomah-Dwamena

**DNA Location** (-20°C) Box 10 I3, Conc.: 40ng/ul; **Tube labeled as:** pCOLA ZISO. T5

**Strain location** (-80°C): Box 20 A9 & A10; **Tube labeled as:** pCOLA ZISO T5 BL21

**Transformed into:** pACCRT-EBP E coli; **Strain location:** Box 20 B9 & B10; **Tube labeled as:** EBP ZISO T5

**NOTE:** This truncated version is non-functional as demonstrated by complementation in E. coli

**Lab Notebook to reference:** Charles Ampomah-Dwamena

**Original clone name (if different):**

**Organism source of gene:** Maize B73

**Cloning vector used:** pColaDuet-1

**Antibiotic markers:** Kanamycin

**Restriction enzyme(s) to release insert:** BamHI and SalI

**Sequence verified:** Yes Junction verified: Yes

**Sequence:**

```
CCATGGGCGACGACGCCCCATCACCATCATCACACCAGCCAGGATCCTGAGGTTTAT
GTTGCTCCTTAACCATAATTGTTCTGTAGTCCATAGTTGATGGAAGCTACCCGGG
AAAGTGGGAGAAAAATAGTTGGGGAGCGTGTATTACCGGTGCTGTCTCGCTGGAAT
TTCACTGCTCCATGAGTTAATCTAATATTGATATACCAATCAATCAATCAATCAATCA
ACCCCATGACATATAATATATAATATAATATAATATAATATAATATAATATAATATAATATA
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*290*
CCAGACATCCACACAGATGGTTGTCAGGTAATTTGTGGTGCCCTTGCCCATACACTATGGATTGGCAACCTCAGTTGCGAGCGGCGCTCTGTCTGCCACCTTTACATCCGCTTCGAATGGTGATGAGGCTGTTGTCACGGGTATGGTGGAAGCCTTGCGAAAAGCTTGCGGCCGCA

GTCGAC

TAA

Note: Histag sequence underlined; start and stop codons in bold


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