A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Genetic Analysis of Recessive Resistance to *Turnip mosaic virus* and Development of Transgenic Plants using *eIF(iso)4E* in *Brassica rapa*

배추의 순무 모자이크 바이러스 저항성 유전연구와 *eIF(iso)4E* 를 이용한 형질전환체의 개발

AUGUST, 2013

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SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

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JULY, 2013

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ABSTRACT

Turnip mosaic virus (TuMV) is one of the major viruses in Brassicaceae crops including Chinese cabbage. The genetic analysis of two Chinese cabbage lines that showed broad spectrum resistance toward TuMV was performed and the recessive resistance gene was named *trs*. An eIF(iso)4E gene was selected as a candidate for the *trs* gene. Among the three copies of eIF(iso)4E (*Braiso4Ea, Braiso4Eb* and *Braiso4Ec*) found in the Chinese cabbage, the *Braiso4Ea* marker (trsSCAR) was perfectly co-segregated with *trs*. But the marker polymorphism was not consistent between resistant lines and susceptible lines, indicating that *Braiso4Ea* is not the corresponding resistance gene. Mapping analysis indicates that the *trs* is located at chromosome A04, between scaffold 000104 and scaffold 040552. The *trs* was highly expected to be one of the *Braiso4Ea* homologs. So *Braiso4Ea* cDNA was used for the further study to confirm its relationship between TuMV resistances. The interaction between eIF4E/eIF(iso)4E and VPg is a critical step in many potyviral infections. To elucidate the key amino acids in the interaction between

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TuMV VPg and eIF(iso)4E, eight amino acids in the cap binding pocket were mutated. The results of yeast two-hybrid and co-immunoprecipitation analysis showed that Trp 95 and Lys 150 amino acid positions are the key amino acids for the TuMV VPg interaction. Complementation of an *eIF4E* knockout yeast strain by mutated eIF(iso)4E proteins showed that all eIF(iso)4E mutants were able to complement eIF4E of yeast, indicating the mutated eIF(iso)4Es retained their function. Transformant analysis was performed to find out if these mutations affect the susceptibility of Chinese cabbage. eIF(iso)4E W95L, W95L/K150E and susceptible wild type were over-expressed in susceptible Chinese cabbage. According to the TuMV screening result of T_1 and T_2 transformants, over-expression of the eIF(iso)4E mutants showed broad spectrum TuMV resistance. In conclusion, this research supports that the eIF(iso)4E of *Brassica rapa* is strongly related to TuMV recessive resistance and single amino acid mutation in the eIF(iso)4E can confer resistance to susceptible plants.

Keywords: *Brassica rapa*, *Turnip mosaic virus* (TuMV), recessive resistance, translation initiation factor, host factor

Student Number: 2008-21260

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LIST OF ABBREVIATIONS

5´ Cap	m^7GTP Cap Structure
BLAST	Basic Local Alignment Search Tool
CAPS	Cleaved Amplified Polymorphic Sequence
cDNA	Complementary Deoxyribonucleic Acid
cM	Centimorgan
Co-IP	Co-Immunoprecipitation
CPRG	Chlorophenolred-ß-D-Galactopyranoside
CTAB	Cetyl Trimethylammonium Bromide
DAS ELISA	Directed Antibody Sandwich Emzyme Linked Immunosorbent Assay
DPI	Days Post Inoculation
eIF4E	Eukaryotic Initiation Factor 4E
eIF4G	Eukaryotic Initiation Factor 4G
EMS	Ethyl Methanesulfonate
ER	Extreme Resistance
EST	Expressed Sequence Tag
ETI	Effector Triggered Immunity

gDNA	Genomic Deoxyribonucleic Acid
GFP	Green Fluorescent Protein
HPT	Hygromycin Phosphotransferase
HR	Hypersensitive Response
HRM	High Resolution Melting
LRR	Leucine-Rich Repeat
NB	Nuclear Binding
NGS	Next Generation Sequencing
ORF	Open Reading Frame
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PepMoV	*Pepper mottle virus*
PTI	PAMP Triggered Immunity
PVY	*Potato virus Y*
RACE	Rapid Amplification of cDNA Ends
RdRp	RNA-dependent RNA polymerase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCAR	Sequence Characterized Amplified Region
SNP	Single Nucleotide Polymorphism
TEV	*Tobacco etch virus*

TILLING	Targeting Induced Local Lesions IN Genomes
TuMV	*Turnip mosaic virus*
UTR	Untranslated Region
VPg	Viral Protein-genome-linked
w/t	Wild-type
Y2H	Yeast Two-Hybrid

GENERAL INTRODUCTION

Plant viruses are one of the most destructive pathogens of many agricultural crops, causing serious yield losses in the world. More than 700 plant viruses are reported to cause devastating diseases (Strange and Scott. 2005). Unlike other pathogens like bacteria and fungi, developing genetic resistance is the best option when dealing with viruses. So far, using natural resistance genes is the most effective and sustainable way to reduce virus disease. The Brassicaceae family, a major group of angiosperms, is well-known for many important vegetable crops that are cultivated in most continents. Cabbage, broccoli, cauliflower, turnip, rapeseed, mustard, radish, horseradish, cress, wasabi, watercress and Chinese cabbage are the main crops. Among these crops, Chinese cabbage (*Brassica rapa*) is commonly cultivated in Asia, Europe and United States. Originated in China and in Korea, Chinese cabbage is the main material for Kimchi, a representative traditional food.

Three major viruses that infect Chinese cabbage are *Turnip mosaic virus* (TuMV), *Cucumber mosaic virus* (CMV) and *Ribgrass mosaic virus* (RMV). *Turnip mosaic virus* (TuMV) infects a wide range of cultivated plant species (318 species in 156 genera of 43 families) and causes significant economic losses in Brassica crops such as Chinese cabbage, turnip, mustard and radish

(Edwardson and Christie. 1991; Shattuck et al. 1992; Shukla et al. 1994). TuMV is transmitted by aphids. The symptoms of TuMV are stunting, mottling of leaves, distorting, black spots and mosaic. The virus especially causes damage on early seedlings. TuMV is a member of the *Potyvirus* genus, the largest genus of plant viruses. It is the only potyvirus that infects Brassica crops (Tomlinson et al. 1987; Walsh and Jenner. 2002). There are several ways to classify TuMV pathotypes. In the 1980s, five strains (C1, C2, C3, C4 and C5) of TuMV were described from Chinese cabbage (Provvidenti et al. 1980; Green and Deng. 1985). In 1996, 12 pathotypes of TuMV were identified from *B.napus* lines (Jenner and Walsh. 1996). In China, seven strains of TuMV (Tu1-7) obtained from ten areas were categorized (Liu et al. 1990).

Resistance genes can be divided into two types, dominant genes and recessive genes. Most of the resistance genes against bacteria and fungi found in the natural environment are the dominant type. However, recessive resistance is found more frequently for plant viruses than for other plant pathogens (Truniger and Aranda. 2009). In addition, potyviruses are well-known for several recessive resistance genes. Most of the TuMV resistance genes are dominant. In *B.napus, TuRB01* (Walsh et al. 1999), *TuRB03* (Hughes et al. 2003), *TuRB04/05* (Jenner et al. 2002, 2003) and *TuRB01b* (Rusholme et al. 2000) were identified. There are only two recessive resistance genes found in *B.*

rapa. Resistance controlled by two genes, a recessive gene (*retr01*) and a dominant gene (*ConTR01*) were found by Rusholme et al. (2007). This showed a broad-spectrum resistance to TuMV, when all the other dominant resistance genes showed a narrow spectrum of resistance to TuMV. By comparison, *retro02* was firstly reported as a single recessive gene that confers TuMV resistance (Qian et al. 2013).

A number of recessive resistance genes to potyviruses correspond to eukaryotic initiation factor 4E (eIF4E) or eIF(iso)4E (Robaglia and Caranta. 2006). The recessive resistance genes, *retr01* and *retr02* are also expected to be related to eIF(iso)4E (Rusholme et al. 2007; Qian et al. 2013).

eIF4E factor initiates translation by binding to the cap of mRNAs and associating with eIF4G. The physical interaction between eIF4E or eIF(iso)4E and the viral genome-linked protein VPg is critical for viral infection by members of the genus *Potyvirus* (Grzela et al. 2006; Miyoshi et al. 2006; Robaglia and Caranta. 2006). The cap binding pocket in eIF(iso)4E seems to be an important spot for the interaction.

This research was performed to analyze a new TuMV recessive resistance gene showing broad spectrum resistance. The study describing a novel *trs* resistance gene was published in 2013. This research also focused on finding eIF(iso)4E key amino acids that abolished the interaction and

developing TuMV resistance in Chinese cabbage. It seems that the eIF4E family plays a significant role in cellular translation and the plant resistance/susceptibility phenotype. Thus, understanding the mechanism of eIF4E or eIF(iso)4E mediated resistance may lead to developing several highly effective virus resistance cultivars.

LITERATURE REVIEW

1. RESISTANCE BREEDING

To control insects, bacteria and fungi, spraying pesticide is the most popular way even in these days. But it is hard to apply this measure to virus diseases. There are not many effective pesticides developed for viruses currently. An alternative way to control virus disease is to use crop cultivars or varieties that are genetically resistant. This is a much more economical way because it can provide sufficient protection with no additional cost implications. It is also environmentally friendly and safe for the consumer (Gómez et al. 2009). The resistance gene can be obtained from wild species of specific crops. Recently, mutagenesis engineering to induce novel resistances became an effective breeding technology. Two types of resistance, dominant and recessive resistance, can exist in plants. The majority of the resistance breeding programs have aimed to introgress dominant genes so far (Pavan et al. 2012). Dominant resistance is often race-specific and can be easily overcome by mutated pathogens (Pavan et al. 2012). Other resistance materials as recessive resistance genes are now being focused.

2. TYPE OF RESISTANCE

1) Non-host resistance

Clearly, most plant species are resistant to most plant viruses. Susceptibility is the exception to the more general condition of resistance or failure to infect, though underlying mechanisms of non-host resistance to viruses are largely unknown and are likely as diverse for viruses as they are for other classes of plant pathogens (Mysore and Ryu. 2004).

2) Host resistance

Host resistance to plant viruses has been more thoroughly investigated. In the case of host resistance, disease symptoms generally are highly localized or are not evident (Kang et al. 2005a).

3. MECHANISMS OF PLANT RESISTANCE TO VIRUSES

More than 80% of reported plant viral resistance is controlled by single genes and the rest are controlled by multiple genes (Kang et al. 2005a). On the basis of inheritance, resistance genes that confer qualitative traits can be classified into two types, dominant and recessive. Slightly more than half of all reported monogenic resistance traits show dominant inheritance (Kang et al. 2005a). Compared to other pathogens, like fungi and bacteria, a relatively high proportion of viral recessive resistance genes have been identified in plants

(Kang et al. 2005a; Truniger and Aranda. 2009). Dominant resistance is often called R gene mediated resistance, and recessive resistance is often called loss-of-function resistance.

1) R genes mediated resistance

R gene-mediated resistance is triggered by direct or indirect interactions between the R gene-encoded protein of the host and the avirulence factor produced by the corresponding avirulence (*Avr*) gene of the invading pathogen (Bonas and Lahaye. 2002; Soosaar et al. 2005). This resistance is associated often with extreme resistance (ER) or a hypersensitive response (HR). R gene mediated resistance has been a good source for conventional breeding so far (Fraser et al. 1990). More than 40 resistance genes showing monogenic dominant inheritance have been cloned (Kang et al. 2005a).

When a plant is invaded by certain pathogens like viruses, the first step of the defense is triggered by the pathogen-associated molecular patterns (PAMPs). This step is termed PAMP-triggered immunity (PTI). PAMPs are conserved compounds of pathogens that can be recognized by plants. The examples are chitin in fungi and flagellin of bacteria (Jones and Dangl. 2006; Bittel and Robatzek. 2007; Boller and He. 2009). This PTI is a basal mechanism that all plants have in common. The next step of plant defense is related to R proteins. R proteins recognize pathogen effectors to establish

effector-triggered immunity (ETI). This recognition event triggers a cascade of defense responses, mediated by a complex-signaling network related to salicylic acid and jasmonic acid. As a result of this resistance procedure, an HR occurs at the site of infection (Robert-Seilaniantz et al. 2007; Bruce and Pickett. 2007; Bari and Jones. 2009). Most of the proteins encoded by R genes have two significant characteristics, a nucleotide-binding (NB) site and leucine-rich repeats (LRRs) (Takken et al. 2006). The direct interaction between R protein and Avr protein is very rare, and it is generally assumed that the most R proteins function in a tripartite module (Van der Hoorn et al. 2002). According to this model, the R proteins interact with a protein which is the target of the Avr protein.

2) Loss-of-function resistance

The information on recessive resistance is much less than that on dominant resistance. Recessive resistance is due to the lack of some specific host factors required to complete virus infection cycle, or due to the mutated host factor (Ruffel et al. 2002; Lellis et al. 2002; Nicaise et al. 2003). This resistance is also called passive resistance (Fraser et al. 1990). In genetic terms, host factors are encoded by dominant susceptibility alleles. So the resistance is thus conferred by recessive resistance alleles (Fraser et al. 1990). Interestingly, all characterized viral recessive resistance genes so far have turned out to encode

isoforms of eIF4E and eIF4G, two components of the translation initiation complex (Kang et al. 2005b; Albar et al. 2006; Robaglia and Caranta. 2006).

In contrast to dominant genes, many recessive genes function at the single cell level or affect cell-to-cell movement (Nicolas et al. 1997; Kang et al. 2005a). Interestingly, more than half of the recessive genes that are identified confer resistance to potyviruses, which is the largest family of plant viruses (Shukla et al. 1994).

Many researchers assumed that recessive resistance may be more durable than dominant resistance (Fraser et al. 1990). The reason for this is that even though viruses that infect plants are quite variable, it seems they use some common host factors. There really are some cases of durable recessive resistance in monocots and dicots which have been widely deployed for more than 50 years (Kang et al. 2005a). Previous studies referred to those critical viral factors that serve as the determinant for pathogenicity in resistance systems. These factors are controlled by recessive genes (Kang et al. 2005a).

4. VIRUS HOST FACTORS

The successful infection by a virus needs a series of diverse interactions between the host and viral factors in every step of its infection cycle. The process includes the expression and replication of the viral genome, cell-to-cell

movement, long distance translocation through the plant vascular system and the suppression of host defense responses (Carrington et al. 1996; Maule et al. 2002). The host factors function for translation of genomic RNAs (Noueiry et al. 2000), coordination of RNA translation/replication (Diez et al. 2000; Noueiry et al. 2003), initiation of negative strand RNA synthesis (Tomita et al. 2003) and RNA replication (Ishikawa et al. 1997; Lee et al. 2001). A number of host factors were also known to play a role in cell-to-cell movement of plant viruses (Gibb et al. 1989; Yoshii et al. 1998a; Yoshii et al. 1998b; Lellis et al. 2002; Ruffel et al. 2002; Gao et al. 2004; Yoshii et al. 2004; Kang et al. 2005b). Compared to this, only few host factors have been shown to be related to systemic movement (Kang et al. 2005a). Only a limited number of host factors have so far been identified.

EIF4E family as a host factor

Eukaryotic initiation factor 4E(eIF4E) is the eukaryotic protein that has an important role in controlling the expression of virtually every protein in the cell (Rhoads et al. 2009). This protein initiates the mRNA translation which is a rate-limiting step of protein synthesis (Jackson et al. 2010).

Eukaryotes mRNA translation is predominantly cap dependent translation. It is a multistep process requiring the assembly of an mRNA–protein complex by different eukaryotic initiation factors. The first step is the

binding of eIF4E to the 5′-7mGpppN-cap of mRNAs. And then, eIF4E recruits eIF4G (the scaffold protein) and eIF4A (the DEAD-box RNA helicase), leading to the formation of the eIF4F protein complex (Goodfellow and Roberts. 2008). The eIF4E and eIF4G complex is also called eIF4F. Protein analysis showed an unusual feature of the eIF4E sequence, the high content of Trp residues. Some of these are involved in the binding of the cap (Joshi et al. 2005) and two conserved Trp residues are involved in the interaction with eIF4G (Joshi et al. 2005). Translation initiates when the eIF4F complex recruits to the small (40S) ribosomal subunit. The subunit carries eIF3 and the ternary initiation tRNA-eIF2-GTP complex through the interaction of eIF3 with eIF4G. Then, the subunit 40S starts to scan the mRNA in the 5A-eIF2-GTP complex through the interaction of eIF3 with eIF4G. The poly (A)-binding protein (PABP) catchs the mRNA ends into proximity for the mRNA circularization. This provides for efficient translation in eukaryotes. The formation of the complete translation initiation complex requires the eIF5B-bridged coupling of the 40S with the 60S ribosomal subunit (Kawaguchi and Bailey-Serres. 2002).

It was already known that all eukaryotes express multiple eIF4E family members. Previously, the study of wheat germ found that there are two versions of eIF4E, termed eIF4E and eIF(iso)4E (Browning et al. 1992). More recently, it was discovered that plants have a second form of eIF4F (eIF4E and eIF4G),

named eIF(iso)4F (eIF(iso)4E and eIF(iso)4G) (Bush et al. 2009). In *Arabidopsis thaliana*, novel cap binding protein (nCBP) was also identified besides the two eIF4E proteins (Ruud et al. 1998).

According to the several studies, functional redundancy has been observed between eIF4E and eIF(iso)4E isoforms under regular growth conditions (Duprat et al. 2002; Lellis et al. 2002; Combe et al. 2005). But there are also examples that describe these different versions of eIF4E having divergent roles either during development or in response to different environmental event (Gallie et al. 1998; Rodriguez et al. 1998; Dinkova et al. 2011). *A. thaliana* has three eIF4E genes and one eIF(iso)4E gene (Robaglia and Caranta. 2006). In contrast with eIF4E, which is differentially subcellularly distributed dependent on the cell type, eIF(iso)4E localizes to both the cytoplasm and nucleus in either quiescent or proliferating cells (Bush et al. 2009).

5. POTYVIRUS FAMILY

The *Potyvirus* genus, which is named after *Potato virus Y* (PVY), is the largest of the 34 plant virus genera and families (Ward and Shukla. 1991). It contains at least 180 definitive and possible members (30% of all known plant viruses) which cause significant losses in agricultural, pasture, horticultural and ornamental crops (Ward and Shukla. 1991). On the 5h end of the viral RNA, a

cap structure, VPg proteins or a 5n phosphate group can be found in different viruses (Thivierge et al. 2005), and in the 3et al. a poly(A) tail, a tRNA-like structure , or a 3′ OH can be found (Thivierge and Nicaise. 2005). The (+) ssRNA of the members of Potyvirus family resemble those of the Picornavirus family, in that they have a VPg (viral protein, genome-linked) at their 5′ end. They have a poly(A) tail at their 3re (A) tado eukaryotic mRNAs. Hence, potyviruses are also called picorna-like viruses. Their positive-sense RNA genomes code for a single polyprotein, which is processed by virus-encoded proteases. Notably, the Potyviral VPg has been identified as an important avirulence determinant several times (Schaad and Carrington. 1996; Nicolas et al. 1997; Keller et al. 1998; Kuhne et al. 2003; Sato et al. 2003).

1) Viral linked genome protein, VPg of members of the Potyvirus family

VPgs from plant and vertebrate viruses share some significant properties. Both groups of proteins are uridylated by virus RNA-dependent RNA polymerase (RdRP) and after that, VPgs act as a primer for complementary viral RNA synthesis (Puustinen et al. 2004; Anindya et al. 2005). The difference between plant virus and vertebrate virus VPgs is that plant VPgs are often viral pathogenicity determinants. Plant virus VPgs are at the center of an intricate protein interaction network made of host and virus proteins (Jiang and Laliberte.

2011). On the other hand, the major function of vertebrate virus VPg is its primer function during complementary viral RNA synthesis (Jiang and Laliberte. 2011). The size of the protein ranges among the plant VPgs from 2 kDa to 22 kDa (Jiang and Laliberte. 2011). Several interacting protein partners of the potyvirus VPg and its precursor forms have been found so far (Jiang and Laliberte. 2011). There were some reports focusing on the interaction between viral RdRP, TuMV VPg and PABP (Wang et al. 2000; Leonard et al. 2004). This interaction seems to facilitate RNA replication. The potyviral VPg especially *Tobacco etch virus* (TEV) and TuMV VPgs have been shown to interact with eIF4E and eIF(iso)4E factors from different hosts. It has been shown that these interactions are necessary for the genome amplification and viral infection (Wittmann et al. 1997; Leonard et al. 2000; Schaad et al. 2000). It is assumed that VPg protein may be important in circularization of (+) ssRNA viruses, which makes it easy to access the host cell translation machinery and enhance the efficiency of translation (Le et al. 1997; Gallie et al. 1998; Borman et al. 2000). It is also considered that the interaction between VPg and the eIF4E family may act in the coupling of translation and replication in (+) ssRNA viruses (White et al. 1992; Novak and Kirkegaard. 1994; Taylor and Carr. 2000).

2) Function of VPg

The 7-methylguanosine 5e 7-m plays an essential role in mRNA translation of eukaryotes (Rhoads et al. 2009). VPg functions as an analogue of the cap structure of mRNA to recruit the translation complex for viral genome translation (Thivierge and Nicaise. 2005; Michon et al. 2006; Beauchemin et al. 2007; Khan et al. 2008). There are some reports describing the competition of viral VPg and 5are so It said the VPg binds to its preferential form of eIF4E more rapidly than does the 5 rcap so that it can inhibits host translation (Plante et al. 2004; Khan et al. 2006; Miyoshi et al. 2006). Other translation related proteins such as eEF1A and PABP were also found in the virus translation/replication complex involving VPg (Beauchemin et al. 2007; Thivierge et al. 2008; Wei et al. 2010). The study using wheat germ extracts showed that the presence of VPgs inhibits the host's cap-dependent translation and stimulates the translation of uncapped IRES-containing viral RNAs (Khan et al. 2008).

Animal viruses tend to shut off host cap dependent translation but in contrast, plant viruses try to use the host translational machinery. This may explain the difference between plant viral VPg and that of the animal virus VPg (Kneller et al. 2006).

3) eIF4E-potyvirus VPg

When the TuMV VPg was used as bait in the yeast two-hybrid assay, eIF(iso)4E of *A. thaliana* was found to be an interaction partner (Wittmann et al. 1997). And after that, another study pointed out that the VPg–eIF(iso)4E interaction is essential to preserve virus infectivity *in planta* (Léonard et al. 2000). Lellis et al. (2002) found three loss-of-susceptibility mutants that have strong TuMV resistance. These mutants have alterations in eIF(iso)4E (Lellis et al. 2002). The actual interaction between VPg and the eIF4E family was also analyzed in many studies. It is shown that the requirement of eIF4E in virus infection is highly virus specific (Nicaise et al. 2007). It turned out that the physical interaction of VPg and eIF4E/eIF(iso)4E correlates with compatible infections in many potyvirus–plant pairs (Gao et al. 2004b; Kang et al. 2005b; Grzela et al. 2006; Yeam et al. 2007; Charron et al. 2008).

According to the previous reports, a few amino acid mutations can abolish the VPg-eIF4E interactions and inhibit virus infection (Yeam et al. 2007; Charron et al. 2008). Most of the amino acid substitutions are located in two regions near the cap recognition pocket (Kanyuka et al. 2005; Robaglia and Caranta. 2006; Charron et al. 2008; Truniger and Aranda. 2009). The virulence determinant was mapped to VPg and most of the critical amino acids are located in the central region that is involved in the interaction with eIF4E (Borgstrom and Johansen. 2001; Duprat et al. 2002; Moury et al. 2004; Grzela

et al. 2006; Bruun-Rasmussen et al. 2007; Roudet-Tavert et al. 2007; Charron et al. 2008; Gallois et al. 2010). It seems that VPg and cap-binding domains of eIF4E overlap to some extent but not perfectly. The pepper pvr1 protein can bind neither VPg nor a cap analog, suggesting that the binding sites might overlap (Khan et al. 2006; Miyoshi et al. 2006; Yeam et al. 2007), but the pvr2 protein, which cannot bind to VPg, still has the ability to bind the cap, suggesting the overlap is not complete (Yeam et al. 2007). Lettuce eIF4E has similar binding affinities for a cap analog and VPg (Michon et al. 2006), but the interactions were shown to occur at different sites (Michon et al. 2006).

Beauchemin et al. 2007 showed that TuMV VPg-Pro interacts with eIF(iso)4E in subnuclear structures *in planta* (Beauchemin et al. 2007). This means that the VPg-eIF4E/eIF(iso)4E complex may have another role of nuclear function disruption. A recent hypothesis is that the VPg-eIF4E/eIF(iso)4E complex could play a role in the coupling process of potyvirus RNA translation and replication (Beauchemin and Laliberte. 2007; Beauchemin et al. 2007; Dufresne et al. 2008; Thivierge et al. 2008). This interaction traps the host replication and translation machineries and sequesters in the cytoplasmic vesicles used for viral multiplication (Truniger and Aranda. 2009). The virus-induced vesicles provide optimal conditions for RNA and protein synthesis, protecting the viral products from host nucleases and

proteases (Aizaki et al. 2004). The virus's translation and the replication of the same RNA templates must be regulated because the 5′ to 3′ movement of ribosome on the RNA conflicts with the 3′ to 5′ activity of the RdRp. The interaction of host and viral factors with each other at both 3′ and 5′ ends may solve this problem (Barry and Miller. 2002; Walter et al. 2002).

6. IDENTIFIED RECESSIVE RESISTANCE GENES

All potyvirus recessive genes involved in plant–virus interactions that have been characterized encode eIF4E, eIF4G and their isoforms. The *pvr2* gene which encodes an eIF4E variant that confers recessive resistance to PVY, became the first natural recessive resistance gene to be cloned in plants (Ruffel et al. 2002). The *pvr2/pvr1* alleles confer resistance to other potyviruses such as *Pepper veinal mottle virus* and TEV (Kang et al. 2005b; Ruffel et al. 2006; Charron et al. 2008). After the characterization of *pvr2*, many candidate gene approaches were performed. Another recessive gene, *pvr6* in pepper corresponds to the eIF(iso)4E locus (Ruffel et al. 2006). Conditional on the presence of *pvr1*, *pvr2* and *pvr6* confer resistance to *Pepper veinal mottle virus* and *Chilli veinal mottle virus* (Ruffel et al. 2006; Hwang et al. 2009; Rubio et al. 2009). The *mo1*, an eIF4E mutant, which confers resistance to *Lettuce mosaic*

virus was also identified (Nicaise et al. 2003). There are other several examples of recessive resistance gene encoding members of the eIF4E/eIF4G family; *pot-1* conferring resistance to PVY and TEV (Ruffel et al. 2005); *rym4* and *rym5* conferring resistance to *Barley yellow mosaic virus* and *Barley mild mosaic virus* (Kanyuka et al. 2005; Stein et al. 2005); *sbm1*-mediated recessive resistance to *Pea seed-borne mosaic virus* (Gao et al. 2004a, b); *nsv* conferring resistance to *Melon necrotic spot virus* (Nieto et al. 2006); *wlv* and *cyv2* conferring resistance to *Bean yellow mosaic virus* and *Clover yellow vein virus*, respectively, which were shown to correspond to the *sbm1* allele (Bruun-Rasmussen et al. 2007; Andrade et al. 2009); and the *bc-3* recessive resistance gene to *Bean common mosaic virus* (Naderpour et al. 2010) is also expected to be an allele of eIF4E. eIF4E and eIF4G show up frequently in recessive resistance studies and Truniger and Aranda (2009) explained the reason. Functional redundancy of several isoforms provides scope for diverse interactions. Interactions between these factors and virus proteins can be quite specific, providing niches for individual viruses (Truniger and Aranda. 2009). Le Gall et al. (2011) commended that the predominant discovery of eIF4E family members in the cloned recessive genes may be due to the fact that the first cloned recessive gene turned out to be eIF4E (Le Gall et al. 2011).

Most of the recessive resistances were expressed in protoplasts and also

seem to affect the cell-to-cell movement of the virus (Díaz-Pendón et al. 2004; Kang et al. 2005b). Thus the resistance is highly effective because it affect the early steps of virus infection. The recessive resistance conferred by eIF4E in pepper is still effective in the field after more than 30 and 50 years from their introduction in agriculture (Lyngkjaer et al. 2000; Kang et al. 2005a). This shows that recessive resistance is somewhat similar to the non-host resistance for it shares the "hallmarks of non-host resistance", durability and broad-spectrum (Humphry et al. 2006). Dominant resistance is highly effective but often race-specific and in most cases, the resistance can be overcome by strains (Pavan et al. 2010). It has been shown that recessive resistance often is durable and broad-spectrum (Pavan et al. 2010).

7. *TURNIP MOSAIC VIRUS*

TuMV is a member of the *Potyvirus* genus (family Potyviridae) and is the only potyvirus known to infect Brassica crops (Tomlinson et al. 1987; Walsh and Jenner. 2002). It has the widest host range of any potyviruses and infects a wide range of cultivated plant species (Edwardson and Christie. 1991; Shukla et al. 1994). Significant economic loss in Brassica crops is due to this virus (Shattuck 1992). In China, it is the most important viral pathogen in some crops such as Chinese cabbage (Walsh et al. 2006).

TuMV was first described in *B. rapa* by Gardner and Kendrick (1921) and Schultz (1921). The wide host range and non-persistent stylet-borne mode of transmission by aphids makes it hard to control (Walsh and Jenner. 2002). Four strains (C1-4) were described from Chinese cabbage in 1980 (Provvidenti 1980) and the fifth strain C5 was identified in 1985 (Green and Deng. 1985). Nineteen TuMV isolates from ten areas of China were identified and seven strains (Tu1-7) were defined with a new set of differentials (Liu et al. 1990**)**. Twelve different pathotypes based on the interactions of 124 TuMV isolates with *Brassica napus* lines were identified in 1996 (Jenner and Walsh. 1996).

8. BRASSICA CROPS

Brassicaceae is an economically important family of angiosperms, which contains approximately 330 genera and about 3,700 species. In this family several well known species are found. *B. oleracea* (broccoli, cabbage and cauliflower), *B. rapa* (Chinese cabbage), *B. napus* (rapeseed, etc.), *Raphanus sativus* (common radish), *Armoracia rusticana* (horseradish), *A. thaliana* (model plants for much scientific research) and many others are included in this family

Chinese cabbage

Chinese cabbage (*B.rapa* subsp. pekinensis) is a crop that is widely cultivated in Asia, especially in Korea, Japan and China. It is also grown in other countries in Europe and North America. In Korea, Chinese cabbage is the major ingredient for Kimchi, a popular traditional food in Korea.

Chinese cabbage is the host for several viruses. In Korea, the most serious viruses are TuMV, RMV and CMV. And in China, TuMV is the biggest problem in Chinese cabbage cultivation these days.

9. TuMV RESISTANCE IN BRASSICA CROPS

Most of the knowledge about resistance in Brassicaceae has been obtained from Arabidopsis plant because it is not easy to obtain information from Brassica crops. This is due to the size and complexities of the genomes (Walsh et al. 2006). Several dominant resistance genes and a few recessive resistance genes have been found in Brassica crops. *TuRB01* (Walsh et al. 1999), *TuRB03* (Hughes et al. 2003), *TuRB04*, *TurB05* (Jenner et al. 2002, 2003), and *TuRB01b* (Rusholme et al. 2000) are the dominant resistance genes found in *B. napus* and *B. Rapa*, but these dominant resistance genes showed a narrow spectrum of resistance to TuMV isolates. The reason that the single dominant resistance genes have a narrow spectrum of resistance may be due to the wide host range and frequent mutation rate of TuMV, allowing the virus to easily

overcome the resistance gene (Tan et al. 2004; Walsh and Jenner. 2006). Rusholme et al (2007) found the combined action of a recessive gene (*retr01*) and a dominant gene (*ConTR01*) showed broad spectrum resistance (Rusholme et al. 2007). The TuMV resistance that is controlled by a single recessive resistance gene, *retr02*, was also found in Chinese cabbage (Qian et al. 2013). Interestingly, *retro1* and the *retr02* are expected to be coincident with an eIF(iso)4E gene (Rusholme et al. 2007; Qian et al. 2013). It seems that the recessive resistance genes have a lot more potential in broad spectrum resistance than dominant genes.

There are some reports of TuMV resistance controlled by quantitative trait loci (QTL). QTLs controlling TuMV-C4 and TuMV–C3 resistance were identified in 2008 and 2009 (Zhang et al. 2008a,b; Qu et al. 2009).

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CHAPTER I

Identification of a broad spectrum recessive resistance gene in *Brassica rapa* and molecular analysis of the eIF4E gene family to develop molecular markers

ABSTRACT

Two Chinese cabbage (*Brassica rapa* L. ssp. pekinensis) lines resistant to TuMV strain CHN5 were identified and found to have broad spectrum resistance against other TuMV strains (CHN2, 3, and 4). Genetic analysis indicated that this TuMV resistance is recessive, and a candidate gene approach was used to identify the resistance gene, which was named *trs* (TuMV resistance discovered at Seoul National University). Based on previous research in Arabidopsis showing that mutations in eIF(iso)4E determine TuMV resistance, the eIF(iso)4E gene was selected as a candidate for the *trs* gene in *Brassica rapa.* Three copies of eIF(iso)4E, *Braiso4Ea*, *Braiso4Eb* and

Braiso4Ec, were amplified and polymorphisms were analyzed between resistant and susceptible lines. Sequence polymorphisms were found in *Braiso4Ea* and *Braiso4Eb*. By contrast, there were no sequence differences in *Braiso4Ec* between resistant and susceptible lines. The linkage analysis showed that the *Braiso4Eb* and TuMV resistance displayed no linkage. A SCAR marker, trsSCAR, was developed using allele specific deletions and SNPs in *Braiso4Ea*. When three F_2 populations were analyzed, the trsSCAR marker perfectly co-segregated with *trs*. However, the presence or absence of the *Braiso4Ea* sequence deletion was not consistent between resistant lines and susceptible lines, indicating that *Braiso4Ea* is not the actual resistance gene. Mapping analysis was performed and the result indicates that the *trs* is located at chromosome A04 between scaffold 000104 and scaffold 040552, demonstrating possibility that *trs* may be another recessive resistance gene tightly linked to *retr02* or another allele. Molecular markers developed in this study will be useful for breeding broad-spectrum resistance.

INTRODUCTION

Turnip mosaic virus (TuMV), a member of the *Potyvirus* genus, has a genome consisting of a positive single-stranded RNA molecule of about 10 kb. A viral protein, VPg (22–24 kDa), is covalently linked to the 5′-end of the genome and a poly (A) tail is present at the 3′-end. TuMV infects a wide range of cultivated plants but the major hosts of the virus are Brassicaceous species, causing severe economic losses in terms of production in Asia, North America and Europe (Edwardson and Christie 1991; Shattuck et al.1992; Mitchell and Bond 2005). TuMV is aphid transmitted in a non-persistent mode of plant virus transmission (Shattuck et al. 1992). The most common symptom of the virus is a distinct mosaic of light and dark green in the leaves. Depending upon the virus strain and the crop species, necrotic streaks, flecks or ring spots may also appear in the infected crops.

Plant virus resistance genes can be dominant or recessive. Most resistance genes are dominant genes that encode R proteins which usually trigger the hypersensitive response. By contrast, recessive resistance more often occurs due to mutation or loss of host factors. Because plant viruses can encode only a few proteins (about four to ten proteins), viral infection is highly dependent on the availability of its host factors (Kang et al. 2005), and viral

susceptibility is often determined by the host factors. Several previous reports showed that eIF4E protein families are major determinants of recessive resistance toward potyviruses (Robaglia and Caranta, 2006), with multiple potyvirus recessive resistance genes identified among eIF4E family members, including *pvr1* in *Capsicum* (Ruffel et al. 2002; Kang et al. 2005a), *mo1* in lettuce (Nicaise et al. 2003), *sbm1* in pea (Gao et al. 2004), *pot-1* in tomato (Ruffel et al. 2005) and *lsp1* resistance alleles created by mutagenesis in *Arabidopsis thaliana* (Lellis et al. 2002; Yoshii et al. 2004). *rym4/5* in barley; which control resistance toward bymoviruses; were also identified as eIF4E (Stein et al. 2005). *retr01* and *retr02* TuMV resistance genes are also supposed to be encoding eIF(iso)4E in Chinese cabbage (Rusholme et al. 2007; Qian et al. 2013).

Chinese cabbage is grown worldwide especially in Asia and Europe. It is used as a vegetable, oilseed and fodder crop. Despite the serious economic loss of Brassica crops caused by TuMV, there has been no report of TuMV resistance genes that could be deployed stably in breeding. TuMV resistance genes that have been identified in Brassicaceae include *TuRB01*, *TuRB03*, *TuRB04* and *TuRB05* in *Brassica napus* (Robbins et al. 1994; Walsh et al. 1999; Hughes et al. 2003; Jenner et al. 2002) and *TuRB01b*, *ConTR01*, *retr01* and *retr02* in *Brassica rapa* (Walsh et al. 2002; Rusholme et al. 2007; Qian et al.

2013). Most of these genes are dominant R genes showing narrow spectra of resistance to specific TuMV isolates. *retr01*, however, is a recessive resistance gene that showed broad spectrum resistance to TuMV strains, together with another dominant gene named *ConTR01* (Rusholme et al. 2007). Recently, there was the first report of a single recessive resistance gene, *retr02,* found in Chinese cabbage (Qian et al. 2013).

It has been shown that eIF(iso)4E of *A. thaliana* is an essential host factor in TuMV infection (Leonard et al. 2000; Lellis et al. 2002; Duprat et al. 2002; Beauchemin et al. 2007; Miyoshi et al. 2008). Recently, there were other reports about the relationship between eIF(iso)4E and Brassica recessive resistance. One of the three copies of eIF(iso)4E in *B. rapa* was identified as likely related to *retr01* (Rusholme et al. 2007), and the gene prediction result of *retr02* also indicated that the allele is coding an eIF(iso)4E protein (Qian et al. 2013).

Here, I describe Chinese cabbage lines that display strong resistance to several TuMV strains and the development of a marker for that resistance. Genetic analysis showed that the TuMV resistance is inherited in a recessive manner. Compared to the previous reports about recessive resistance of *Brassica rapa*, the gene I discovered is a single recessive gene that controls

resistance to four different strains of TuMV. Since eIF(iso)4E was identified as a potyvirus resistance candidate gene, I analyzed this gene family in more detail.

MATERIALS AND METHODS

Plant materials and virus strains

Brassica rapa subsp. pekinensis 'Samjin' (Monsanto Korea, Chochiwon) was used as a susceptible control and for the cloning of susceptible eIF(iso)4E cDNA. Chinese cabbage lines (SB15, SB16, SB17, SB18, SB20, SB22, SB23, SB24 and SB25) were provided by the National Institute of Horticultural and Herbal Science and were used for the extraction of eIF(iso)4E genomic DNAs and RNAs. Another susceptible Chinese cabbage cultivar GJS2A was provided by Hankook Seed. Co., Ltd. It was used for the F_2 population construction. The susceptible Chinese cabbage lines SB20, SB24, GJS2A and resistant Chinese cabbage lines SB18, SB22 were crossed to get F_1 plants. F_1 plants were self-pollinated to generate the F_2 populations. A total of 57 F_2 individuals from SB22 and SB24 parents, 71 F_2 individuals from SB18 and SB20 parents, and 155 F_2 individuals from GJS2A and SB18 parents were used for genetic analysis. TuMV CHN2, 3, 4 and 5 were provided by Namhan Huh (Nongwoo Bio, Yeojoo, Korea). Virus inoculum was propagated in susceptible Chinese cabbage cultivar 'Samjin'. TuMV CHN2, 3, 4 and 5 were used to test the resistance spectrum of Chinese cabbage lines. The TuMV CHN5 strain was used for the F_2 screening (Green and Deng 1985; Liu et al. 1990).

Virus screening procedure

Plants were inoculated at the two-to-four leaf stages by mechanical inoculation. Virus inoculum was prepared by grinding TuMV-inoculated leaves in 50 mM potassium phosphate buffer (pH 7.5). Mechanical inoculation was carried out by applying virus inoculum with light Carborundum dusting. Plants were monitored daily after 20 days post inoculation (dpi) and classified as resistant or susceptible to TuMV by the absence or presence of visual virus symptoms. Susceptibility or resistance was confirmed by DAS-ELISA using TuMV antibodies (Kisan Biotech Co. Ltd, Seoul, Korea). The plants were scored as susceptible when the ELISA absorbance value was higher than 2.5 times the mean absorbance value of three un-inoculated samples.

Cloning and sequence analysis of eIF(iso)4E

Total RNA was isolated from Chinese cabbage using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Total genomic DNA was isolated from young green leaves of Chinese cabbage using the CTAB method of Hwang et al. (2009). Full length eIF(iso)4E sequences were amplified by RT-PCR (reverse transcription polymerase chain reaction) with gene-specific primers (Table 1). PCR to amplify cDNAs and gDNAs was performed in 50 μl reaction volumes with 50-100ng of DNA as template, 1 x PCR buffer (Takara Shuzo Co., Kyoto,

Japan), 2.5 mM dNTP, 1.25 units of EX-Taq (Takara Shuzo Co., Kyoto, Japan) and 5 pmol of each primer. The PCR conditions were 94^{o}C for 30 s, 55^{o}C for 30 s, 72^{o}C for 1 min, for 30 cycles. DNA fragments were cut from the gel and DNA was recovered using a Zymo Gel Recovery kit (Zymo Research, Orange, CA, USA). PCR products were cloned using a TOPO TA Cloning Kit (with pCR2.1-TOPO vector, Invitrogen Life Technologies, Carlsbad, CA, USA), and sequencing was performed at NICEM (Seoul National University, Seoul, Korea) by the Sanger method.

For 3′ rapid amplification of cDNA ends (3′ RACE), cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) using the oligo (dT)-containing 3sized using SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) using the oligo OrThe 3′ - RACE fragments were purified and cloned into T-blunt vector (Sorgent Co., Daejeon, Korea) and then sequenced.

Phylogenetic analysis of the eIF4E family in Brassica was performed using MEGA Program V5 (Tamura et al. 2007) with 1,000 bootstrap replications.

Developing eIF(iso)4E molecular markers

The eIF(iso)4E gene was used as a TuMV resistance marker in Chinese cabbage. A National Center for Biotechnology Information (NCBI) BLAST nucleotide search was performed in advance. Primers were designed based on eIF(iso)4E gDNA sequence alignment data using the *B. rapa* accessions HM131209.1 (*BraA. eIF(iso)4E a*), HM131211.1 (*BraA. eIF(iso)4E c*) and HM131210.1 (*BraA. eIF(iso)4E b*).

For the *Braiso4Ea* association analysis, a SCAR (Sequence Characterized Amplified Region) marker was developed. Three marker primers were designed based on sequence alignment of *Braiso4Ea-1* and *a-2*. PCR was performed in 25 μl reaction volumes with 50-100 ng of gDNA as template, 1 x PCR buffer containing 1.56 mM $MgCl_2$, 62.5 mM KCl, 12.5 mM Tris-Cl (PK science., Seoul, Korea), 2.5 mM dNTP, 0.2 unit of home-made Taq DNA polymerase purified as previously reported (Desai et al. 1995), 6 pmol of primer F1, 2 pmol of primer F2 and 5 pmol of primer R (Table 1). The PCR conditions were 94^{o}C for 30 s, 60 ^{o}C for 30 s, 72^{o}C for 1 min 40 s, for 37 cycles. To analyze the co-segregation of *Braiso4Eb, Braiso4Eb* was amplified with gene-specific primers and digested with *XbaI*, based on the single nucleotide polymorphisms between susceptible and resistant sequences.

Linkage analysis and resistance gene mapping

The scaffold 000104 and its nine neighborhood scaffolds were previously mapped at the chromosome A04 (Wang et al. 2011; Qian et al. 2013). Primers were designed at these ten scaffolds including scaffold 000104 and their PCR products were sequenced. gDNA of SB18, GJS2A, SB22 and SB24 were used for the sequencing, and the sequences were aligned to find SNPs for HRM marker development. Ten scaffolds anchored in chromosome A04 were partially sequenced to develop SNP markers. SNPs on these scaffolds were identified and marker primers were designed to amplify DNA fragments including SNPs (Table 1). The high resolution melting (HRM) method was used to analyze molecular markers. HRM was performed in 20 μl reaction volumes with 125 ng of gDNA as template, 1 x PCR buffer containing 1.25 mM MgCl2, 50 mM KCl, 10 mM Tris-Cl (PK science., Seoul, Korea), 2.5 mM dNTPs 1.25 μM SYTO9 (Invitrogen Life Technologies, Carlsbad, CA, USA), 10 pmol of reverse and forward primers, 0.2 unit of home-made Taq DNA polymerase using a Rotor-gene TM 6000 thermocycler (Corbette Research, Sydney, Australia). Cycling conditions were 95^{o}C 4 min followed by 55 cycles of 95^{o}C 15s, 50^{o}C 15s and 70 ^{o}C 30s, holding at 95^{o}C 1 min and 40^{o}C 1 min. HRM was analyzed for each 0.1^{o}C increment between 70 and 95^{o}C.

Linkage analysis of markers developed for the *trs* mapping was

performed using two F_2 populations, GJS2AxSB18 and SB22x24. Linkage between SNP markers and the *trs* locus was established using Carthagene software (de Givry et al. 2005). The Kosambi function was applied to convert recombination fractions into map distances.

Colony analysis for sorting eIF(iso)4E copies

HRM analysis was used to screen the colonies containing 3´ RACE product clones. PCR was performed in 20 µl reaction volumes with template, 1x PCR buffer containing 1.25 mM $MgCl_2$, 50 mM KCl, 100 mM Tris-Cl, 2.5 mM dNTPs and 1.25 µM SYTO9 (Invitrogen Life Technologies, Carlsbad, CA, USA). Intact bacterial cells were re-suspended in the PCR mixture. Cycling and HRM conditions were 95^{o}C 4 min followed by 50 cycles of 95^{o}C 15s, 55^{o}C 15s and 70 ^{o}C 30s, holding at 95^{o}C 1 min and 40^{o}C 1 min. HRM was analyzed for each 0.1 ^{o}C increment between 70 and 95^{o}C.

Table 1. Primers used in this study.

Primer	Direction	Primer sequence	Position
eIF(iso)4E	F	ATGGCGACAGAGGATGTGAA	Exon 1
	R	TCAGACAGTGAACCGAGTTC	Exon 5
eIF(iso)4E b UTR	F	ATCTCTCTCTGATTCACAAGTAGCGAAGAAT	Intron 1
	R	GACATCTTTCATCTTCCTCTAGCGTCTTCGTT	Intron 5
eF(iso)4E c	R	TAGACACTAAATCGACTT	Exon 5
eIF(iso)4E a	F	TTCGACACCGTCCAAGACCTCTGGGGG	Exon 1
	R	CGGCGTCAGTTTGCTAGGGATG	Exon 2
eIF(iso)4E 3´ UTR	R	GTCCAAGATCAAGCTGATCAAC	UTR
trsSCAR marker	F1	ATTTATTGTTAGCGATCTGTGGTTTCCT	Intron 1
	F2	GATGTCCCATTATTGTTTGATTATCCTGTTCC	Exon 3
	R	GAAACAAGGTAGTTATCCATTCTAAGTGTG	Intron 3
Sca552[a] 1-2 HRM 2Ax18	F	AAATATAATCTAAAGGTTCATCAACAC	28.2kb
	R	ACAAGGAATTCAAGAAAAGATGGA	
Sca177[b]-1 HRM 2Ax18	F	TTGGGAGTAGGTGTGTAAGAACGT	4.7kb
	R	CTAAGAGGTTGGTTCTTACACTG	
Sca104[c] 4-2 HRM 2Ax18	F	CATCGCAGACATTTTAGGAGGT	552kb
	R	ACTGGTACTCTTTTATCTTGCA	
Sca104-5 HRM 2Ax18	F	CTTTGGCTTCATCAGGTCCTT	12.4kb
	R	CAAACCACTTCATGCTGTTTTCAAAC	
Sca83[d] 1-7 HRM 2Ax18	F	CGCGTCTTGTTCAGAAACATGAG	318kb
	R	CCACATTTGGACATGAAGTTCTT	
Sca83 1-2 HRM 22x24	F	CCATAAAACATCAGTATTACAC	56.2kb
	R	GGATAATAGGCATCTACGAAATTAAG	
Sca104 10-5 HRM 22x24	F	TTGGTTACCGCAGTTTAAGTTTCC	754.7kb
	R	CTGATGCCTTTTGCAAATTGTCGT	
Sca104-1 HRM 22x24	F	GCTCTGATTCTCTGCATCACCA	543.2kb
	R	CAGCTATTCAGAGAAGTCTTTGAGG	
Sca552 2-8 HRM 22x24	F	ATGGTCGCTGAGATTAGAGGAT	403.2kb
	R	CTCATCCGATAATAATTTTGATTTATC	

[a] Scaffold 040552
[b] Scaffold 000177
[c] Scaffold 000104
[d] Scaffold 000083

RESULTS

TuMV screening to select virus-resistant Chinese cabbage lines

Chinese cabbage lines (SB15, SB17, SB18, SB20, SB22, SB23, SB24 and SB25) that were presumed to have recessive resistance based on previous reports of the National Institute of Horticultural and Herbal Science were screened for TuMV resistance. Chinese cabbage cultivar 'Samjin' was used as a positive control because this cultivar is known to be highly susceptible to TuMV. Virus resistance or susceptibility was assessed by both visual symptoms and ELISA absorbance values. A line was considered susceptible when the inoculated plants showed typical symptoms, such as a mosaic of light and dark green, dwarf symptoms, necrosis, and puckering. After 20 to 30 days dpi with TuMV CHN5, obvious typical TuMV symptoms were observed on the upper leaves of Samjin (data not shown). Six Chinese cabbage lines (SB15, SB17, SB20, SB23, SB24 and SB25) were classified as susceptible according to their symptoms. SB15, SB17, SB20 and SB24 showed strong mosaic symptoms and other significant TuMV symptoms. Symptoms in SB23 and SB25 were also obvious but were relatively weak compared to those of the other four susceptible lines. Two lines, SB18 and SB22, showed no visible symptoms on both inoculated and upper leaves. The ELISA values of four Chinese cabbage

lines (SB15, SB17, SB20 and SB24) were similar to those of the 'Samjin' positive control. In accordance with their weaker symptoms, the ELISA values of SB23 and SB25 were relatively low compared to those of the positive control and the other four susceptible lines. The ELISA values of two lines, SB18 and SB22, were the same as mock inoculated control (Figure 1A).

The resistance spectra of these selected plants were evaluated using TuMV-CHN2, 3, 4 and 5. CHN2 and CHN3 induced mild symptoms compared to CHN4 and CHN5 strains in susceptible lines and Samjin (data not shown). However, there were no differences in the resistant lines, SB18 and SB22. No symptoms appeared in these plants, even in inoculated leaves. ELISA data also confirmed that the two Chinese cabbage lines were all highly resistant to four TuMV strains (Figure 1D). The ELISA values of these two lines were uniformly low upon inoculation with any of the four TuMV strains, similar to mock inoculated controls.

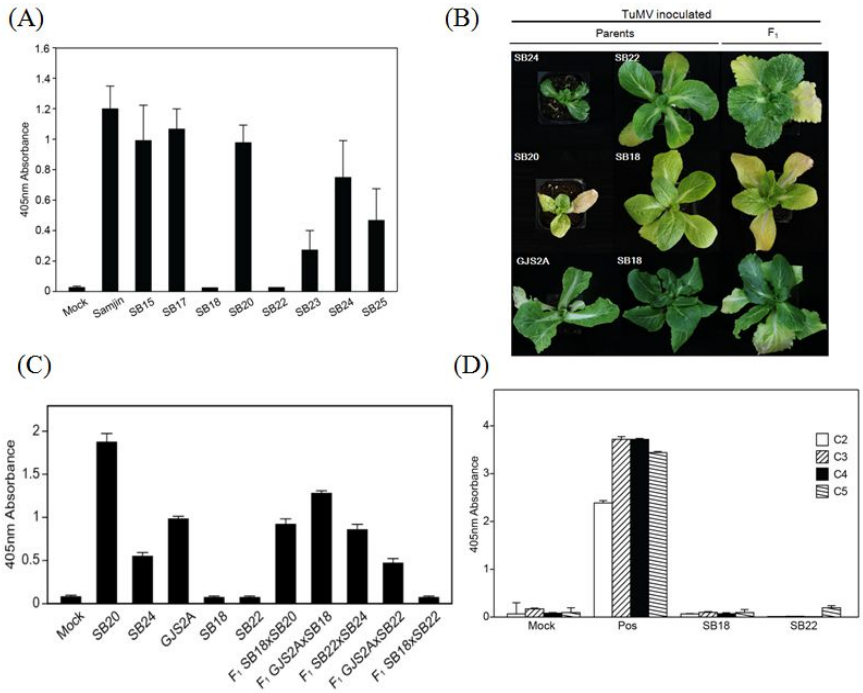


Figure 1. Virus resistance screening of Chinese cabbage lines. (A) Enzyme-linked immunosorbent assay. SB15, 17, 18, 20, 22, 23, 24 and 25 lines were screened. DAS-ELISA was performed at 25 dpi to test the virus accumulation. (B) Pictures of Chinese cabbage plants inoculated with TuMV CHN5. The plants were photographed at 57 dpi. SB24, TuMV-susceptible paternal parent SB24; SB22, TuMV-resistant maternal parent SB22; F_1, susceptible F_1 individual; SB20, TuMV-susceptible paternal parent SB20; SB18, TuMV-resistant maternal parent SB18. GJS2A, TuMV-susceptible paternal parent GJS2A. (C) Virus accumulation of two resistant parents SB18 and SB22, three susceptible parents SB20, SB24 and GJS2A and their five F_1 combinations SB18xSB20, GJS2AxSB18, SB22xSB24, GJS2AxSB22 and SB18xSB22. DAS-ELISA was performed at 32 dpi to test the virus accumulation. (D) Resistance spectrum of SB18 and SB22. Four TuMV strains, CHN2, 3, 4 and 5 referred as CHN2, 3, 4 and 5 were used for screening. Mock, negative control; Pos, positive control line 'Samjin'. Error bars represent standard deviation.

Inheritance of resistance to TuMV in F_2 populations

To determine the inheritance pattern of TuMV resistance, resistant Chinese cabbage lines SB18 and SB22 were crossed with susceptible lines SB20, SB24 and GJS2A (TuMV susceptible line provided by Hankook seed. Co., Ltd) to obtain F_1 seeds. F_1 plants were then self-pollinated to generate three F_2 populations, SB18x20, GJS2AxSB18 and SB22x24. Parental lines, and the F_1 and F_2 plants were screened with TuMV strain CHN5. All F_1 plants showed susceptibility, which indicates that the resistance gene may be the recessive gene (Figure 1B, C). However, the symptoms were milder than those of the susceptible parents, as shown in Figure 1. Stunting and puckering symptoms were much weaker in F_1 than in the susceptible parents (Figure 1B). A total of 71 F_2 plants of SB18x20, 155 F_2 plants of GJS2AxSB18 and 57 plants of SB22x24 were screened for the phenotypic segregation analysis. In the SB18x20 F_2 population, 63 plants were susceptible and 8 plants were resistant (Table 2). The segregation ratio of resistance in the F_2 population greatly deviated from a monogenic ratio of resistant to susceptible plants (χ^2 =7.141; $P < 0.05$). Hence, GJS2AxSB18, another F_2 population derived from SB18, was screened. In the population, 114 individuals were susceptible and 41 plants were resistant. Therefore, the segregation ratio of resistance in the F_2 population also fitted with a monogenic 1:3 ratio of resistant to susceptible plants (χ^2

=0.174; P = 0.676) (Table 2). In the SB22x24 F_2 population, 43 plants were susceptible and 14 plants were resistant. Therefore the segregation ratio of resistance in the F_2 population fitted with a monogenic 1:3 ratio of resistant to susceptible plants (χ^2 =0.006; P = 0.938) (Table 2). Based on these results, I concluded that the resistance of SB18 and SB22 is controlled by a single recessive gene. This resistance gene was named *trs* (TuMV resistance discovered at Seoul National University).

Table 2. Genetic analysis of TuMV resistance using three F_2 populations.

F_2 population	TuMV strain	No. of F_2 plants	Phenotype (R:S)	Expected ration (R:S)[a]	χ^{2b}	P[c]
SB18(R)x20(S)	CHN5	71	8:63	1:3	7.141	0.0075
SB22(R)x24(S)	CHN5	57	14:43	1:3	0.006	0.938
GJS2A(S)xSB18(R)	CHN5	155	41:114	1:3	0.174	0.676

[a] Resistant plants(R) versus susceptible plants (S)
[b] Chi-square test
[c] Probability value

Allelism test of SB18 and SB22 TuMV resistances

Chinese cabbage lines SB18 and SB22 all showed strong resistance against TuMV. Inheritance data indicate that these resistance genes were a single recessive resistance gene. To confirm the allelism of the resistance genes of SB18 and SB22, resistant x resistant and resistant x susceptible crosses were made. Five to seven F_1 progenies of each cross (SB18x22, SB18x20, GJS2AxSB18, SB22x24 and GJS2AxSB22) were inoculated with TuMV CHN5 with their parents. Virus resistance or susceptibility was assessed by both visual symptoms and ELISA absorbance values at each 15, 22 and 32 dpi. F_1 SB18x20, GJS2AxSB18, SB22x24 and GJS2AxSB22 showed TuMV susceptibility as expected, but the F_1 seedlings from the cross between SB18 and SB22 resistance lines showed uniform resistance (Figure 1C). The symptom development in the parents was 7-10 days faster than that of the F_1 plants. At 40 dpi, all the inoculated F_1 plants derived from SB18x22 showed no visible symptoms whereas other F_1 plants showed typical TuMV symptoms (Figure 2). The ELISA values confirmed the result (Figure 1C). This result demonstrated that the TuMV resistance genes of SB18 and SB22 are controlled by the same gene, or very tightly linked genes.

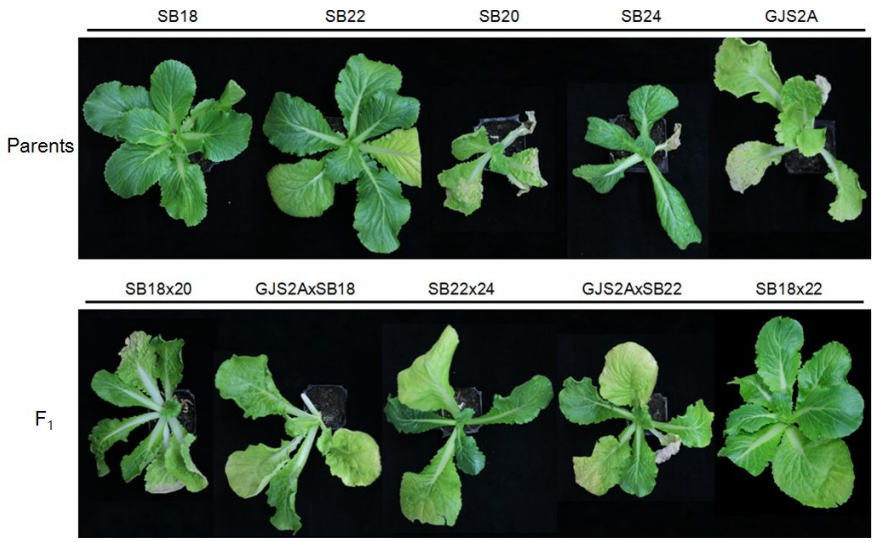


Figure 2. Pictures of Chinese cabbage plants tested for allelism test. The plants were inoculated with TuMV CHN5. These were photographed at 40 dpi. Upper pictures represent the resistant parents SB18, SB22 and the susceptible parents SB20, SB24 and GJS2A. Lower pictures represent five F_1 plants. SB18, TuMV-resistant maternal parent SB18; SB22, TuMV-resistant maternal parent SB22; SB20, TuMV-susceptible paternal parent SB20; SB24, TuMV-susceptible paternal parent SB24; GJS2A, TuMV-susceptible paternal parent GJS2A. The five F_1 combinations are SB18xSB20, GJS2AxSB18, SB22xSB24, GJS2AxSB22 and SB18xSB22.

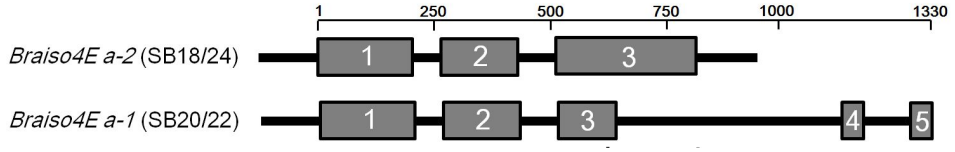
DNA sequence analysis of eIF(iso)4E copies

Because mutations in eIF(iso)4E resulted in TuMV resistance in Arabidopsis (Lellis et al. 2002; Yoshii et al. 2004), I chose eIF(iso)4E as a candidate gene for *trs*. To analyze its genomic DNA sequence, gDNA from resistant and susceptible lines was extracted and eIF(iso)4E was amplified using several specific primers (Table 1).

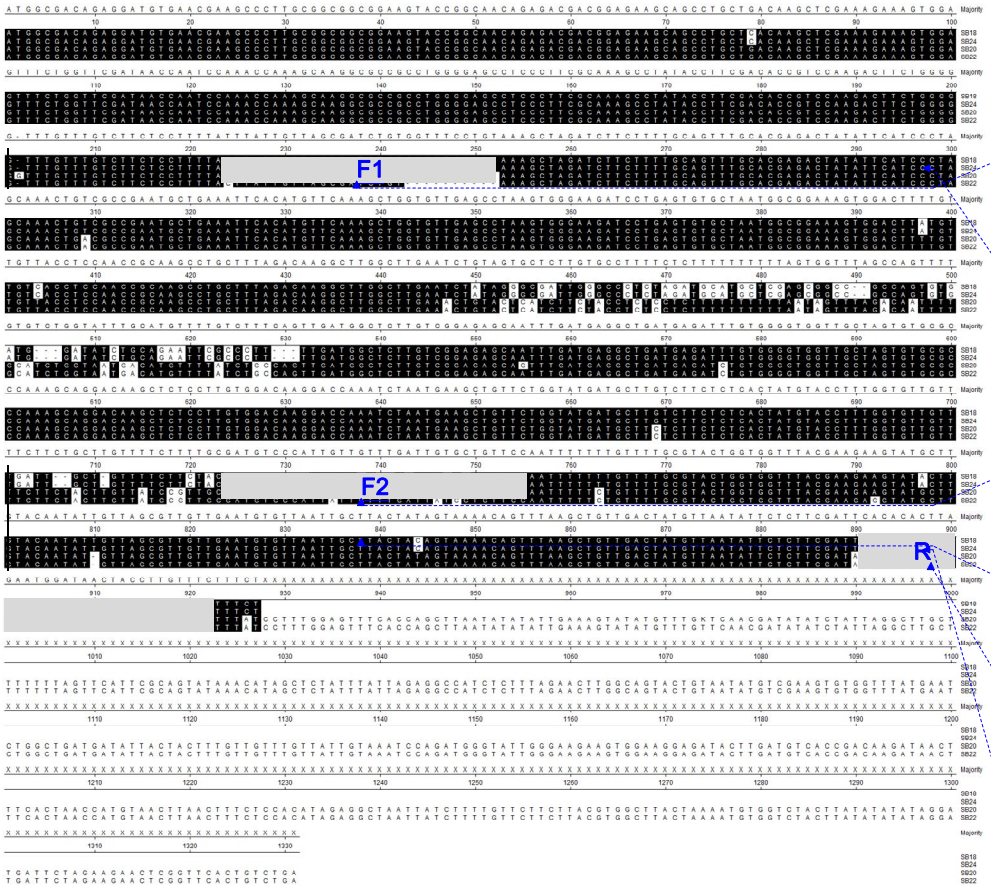
Cloned gDNA was sequenced and three copies of the eIF(iso)4E gene were found. The identified eIF(iso)4E gene copies were named *Braiso4Ea*, *Braiso4Eb* and *Braiso4Ec* to distinguish them from the NCBI reference sequences. The copy named *Braiso4Eb* showed 99% identity to *BraA. eIF(iso)4E b*. The gene copy named *Braiso4Ec* had 96% identity to *BraA. eIF(iso)4E c* (NCBI BLAST, http://www.ncbi.nlm.nih.gov/BLAST/). There were two alleles of the *Braiso4Ea* gene copy. These two alleles, named *Braiso4Ea-1* and *Braiso4Ea-2*, shared 96% and 97% identity with *BraA. eIF(iso)4E a*, respectively. However, the two alleles have many differences in terms of single nucleotide polymorphisms and 3′ end sequences. I found that SB20 and SB22 have the *Braiso4E a-1* allele, whereas SB18 and SB24 have the *Braiso4Ea-2* allele (Figure 3). The other susceptible Chinese cabbage lines SB15, SB17, SB23, SB24 and SB25 all have the *Braiso4E a-1* allele as SB20 (data not shown). The genome structures of the two *Braiso4Ea* alleles are quite

different. Exons 4 and 5 of *Braiso4Ea-1* are missing in *Braiso4Ea-2*. Sequence polymorphisms were found mostly in introns rather than exons, with the most polymorphic region being 445 bp-545 bp, in intron 2. Few single nucleotide polymorphisms (SNPs) were discovered in exons but none of these SNPs showed consistency in resistant and susceptible lines. In cDNA and amino acid alignments, *Braiso4Ea-2* was found to have an early stop codon in exon 3 compared to *Braiso4Ea-1* (Figure 4). Exon 3 of *Braiso4Ea-2* is approximately 200 bp longer than that of *Braiso4Ea-1* because part of the intron is retained, possibly due to incomplete splicing. As the conserved exon parts (exon 4 and 5) are missing in *Braiso4Ea-2,* the allele may not be functional. These two alleles of *Braiso4Ea* were used to perform an association study of TuMV resistance.

(A)



(B)



서식 있음: 글꼴: (영어) Arial Unicode MS, (한글) Arial Unicode MS, 굵게

서식 있음: 가운데

서식 있음: 글꼴: (영어) Arial Unicode MS, (한글) Arial Unicode MS, 굵게

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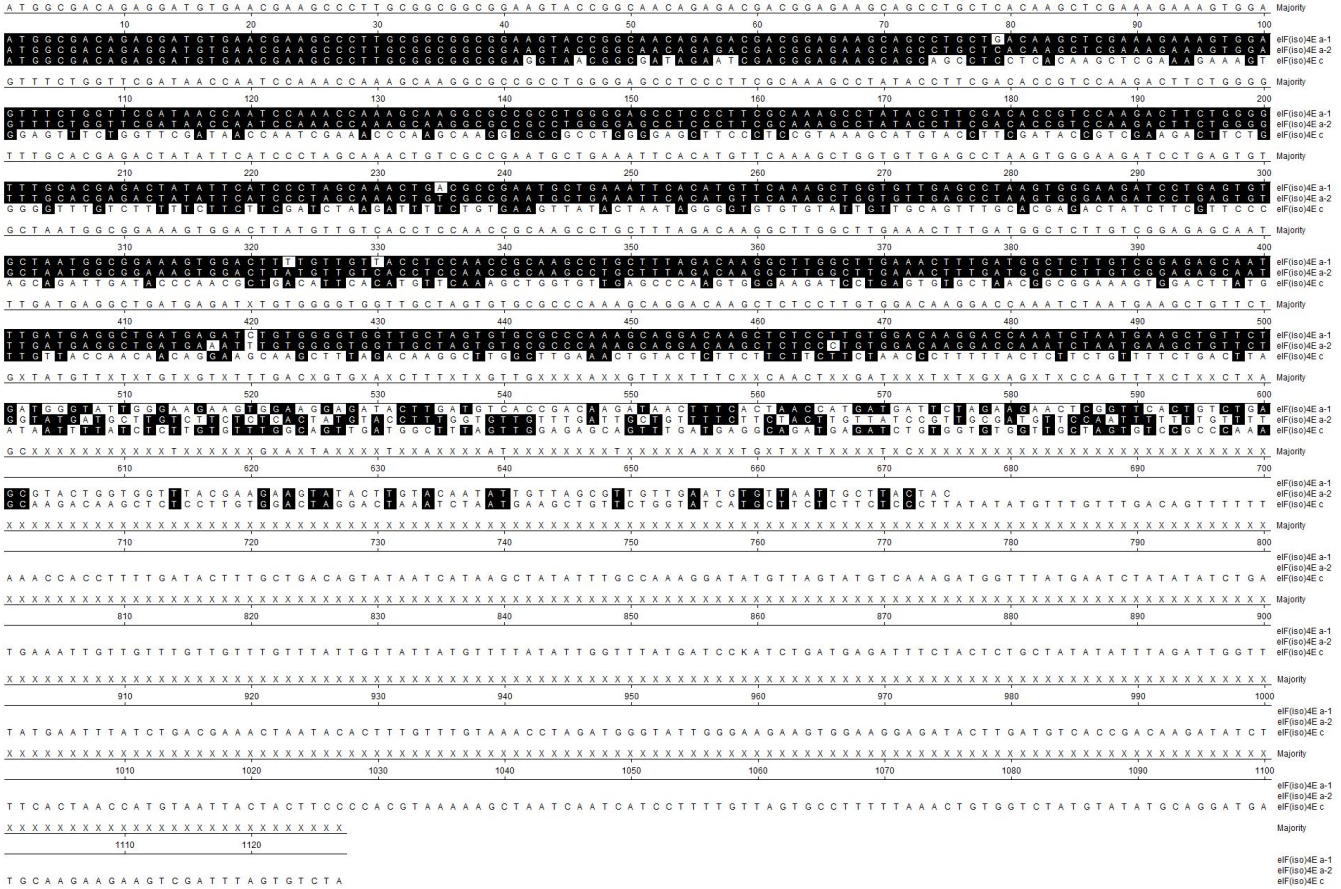
서식 있음: 글꼴: (영어) Arial Unicode MS, (한글) Arial Unicode MS, 굵게

서식 있음: 가운데

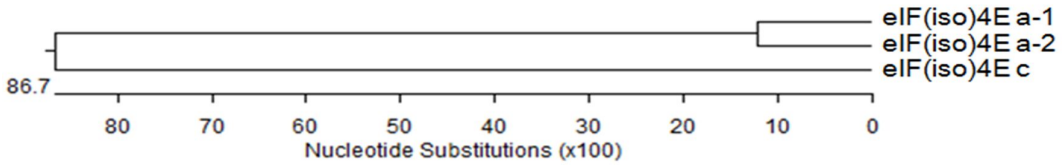
서식 있음: 글꼴: (영어) Arial Unicode MS, (한글) Arial Unicode MS, 굵게

Figure 3. Comparison of the *Braiso4Ea* alleles. (A) Structure of the genes indicating positions of exons, introns, and generic primers used for marker. Exons are indicated by black boxes and introns are thin lines. The size of each sequence is labeled above the structure. (B*)* Sequence alignment of the gDNA sequences of *Braiso4Ea-1* and *Braiso4Ea-2* from SB20, SB22 and SB18, SB24. The trsSCAR marker sites based on allele specific deletions and SNPs are indicated using gray shadow box. F1 is based on *Braiso4Ea-2* polymorphisms and F2 is based on *Braiso4Ea-1* polymorphisms. R is the common reverse primer for F1 and F2.

(A)



(B)



(C)

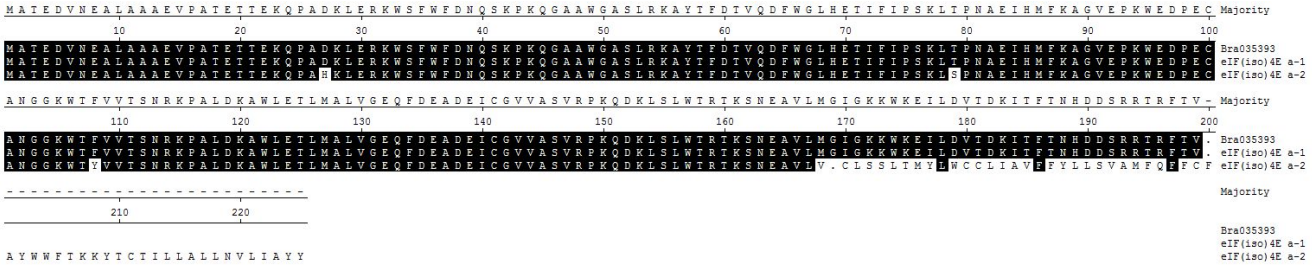
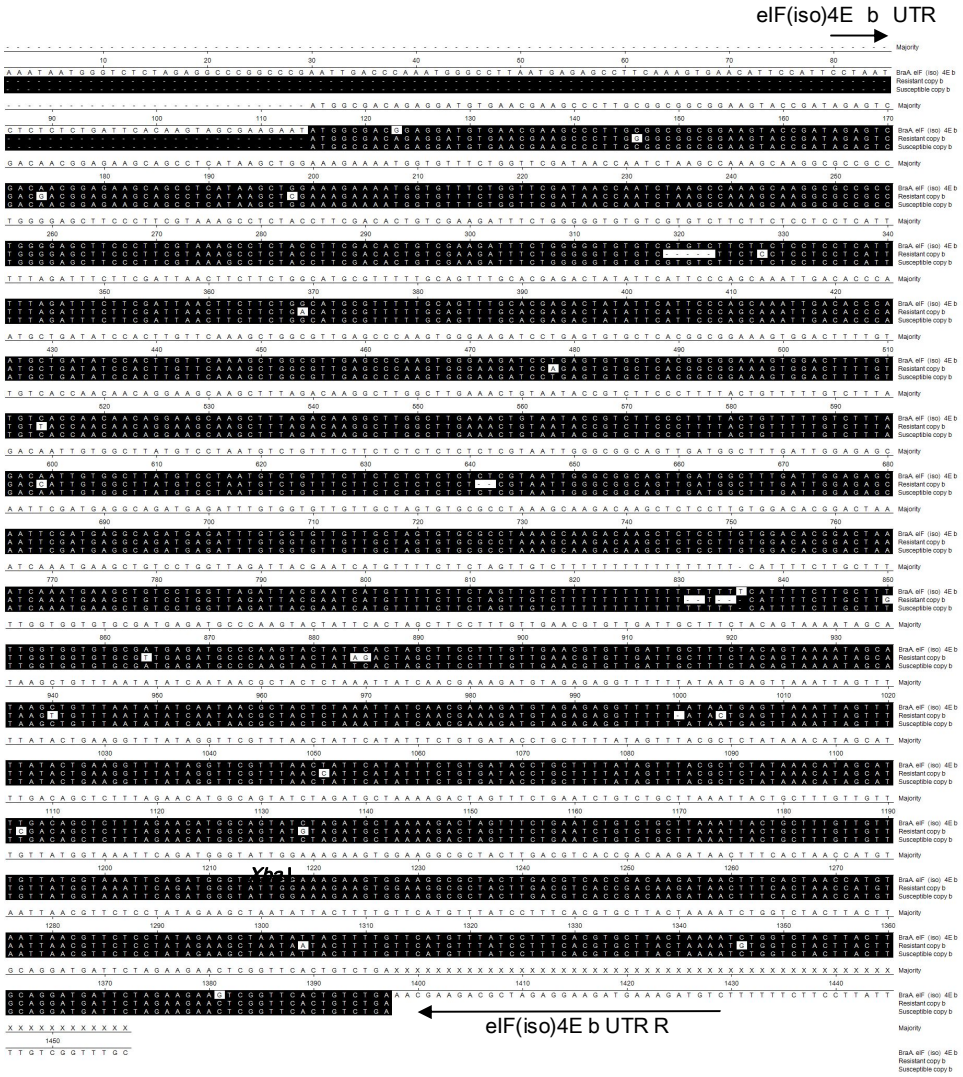


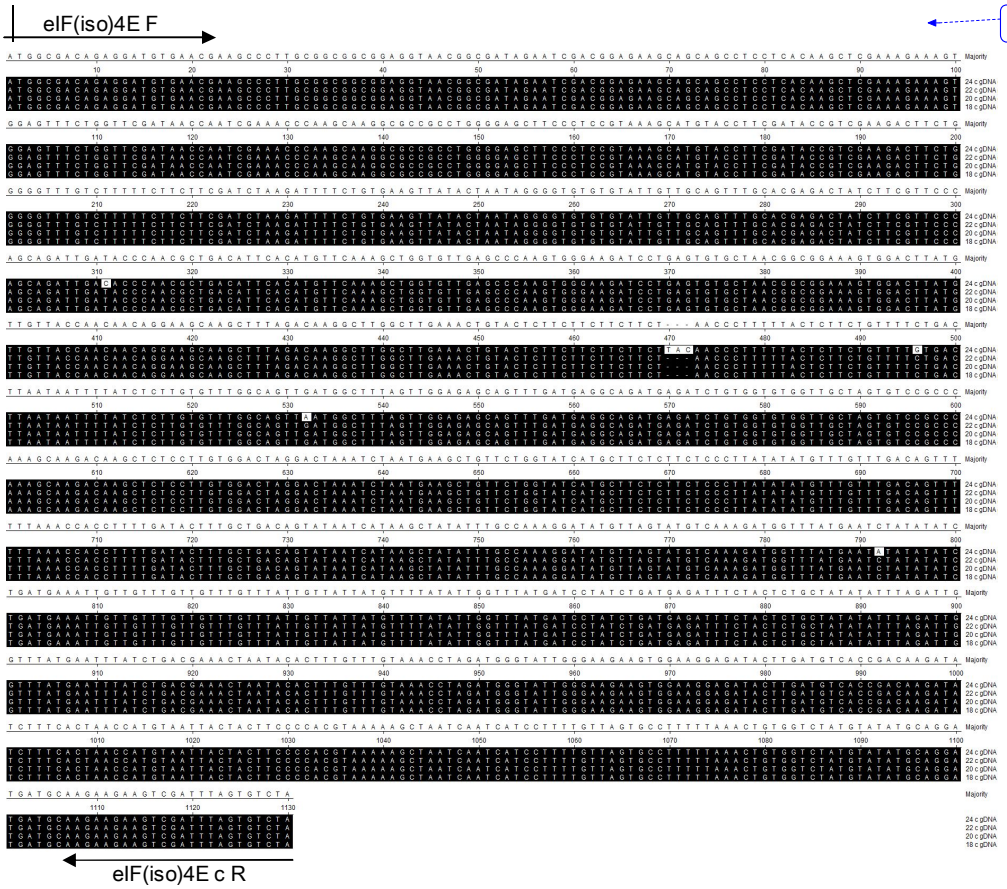
Figure 4. Expressed cDNA copies of *B. rapa*. (A) Sequence alignment of the cDNA sequences of *Braiso4Ea-1, a-2* and *Braiso4Ec*. (B) Phylogenetic tree based on alignment of the cDNA sequences of eIF(iso)4E. (C) Amino acid alignment of *Braiso4Ea-1*, *Braiso4Ea-2* and Bra035393 of scaffold 000104. eIF(iso)4E a-1, *Braiso4Ea-1;* eIF(iso)4E a-2, *Braiso4Ea-2;* eIF(iso)4E c, *Braiso4Ec.*

The *Braiso4Eb* and *Braiso4Ec* copies from each line were aligned (Figure 5). SNPs between susceptible and resistant *Braiso4Eb* copies were detected in the sequence alignment. These SNPs were used for further analysis of TuMV resistance association. By contrast, there were no sequence variations in *Braiso4Ec* sequences among four Chinese cabbage lines.

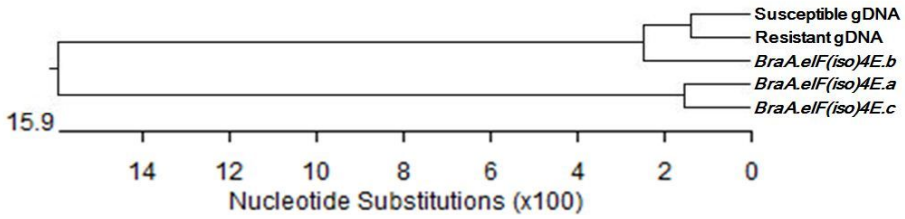
(A)



(B)



(C)



(D)

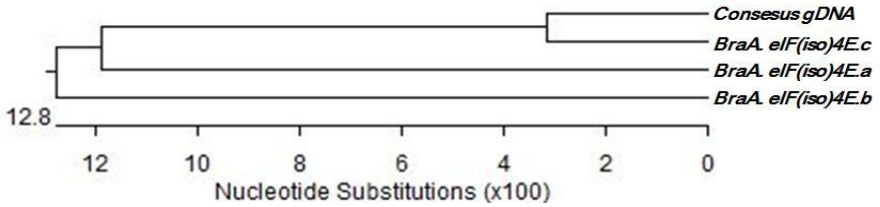


Figure 5. gDNA sequences of *Braiso4Eb and c*. (A), (B) Sequence alignment of the gDNA sequences of *Braiso4Eb* (A) and *Braiso4Ec* (B) of resistant and susceptible lines. *Xba1* RE site marked above the sequence is the marker site for the eIF(iso)4E association analysis. Primer site is shown with arrows. (C), (D) Phylogenetic trees based on alignment of the gDNA sequences of *Braiso4Eb* (C) and *c* (D).

TuMV resistance linkage analysis of *Braiso4Ea* and *Braiso4Eb*

To test which eIF(iso)4E gene copy is related to the recessive resistance, linkage analysis of each eIF(iso)4E gene copy was performed. As *Braiso4Ec* did not show specific sequence variation among four susceptible and resistant Chinese cabbage lines, only *Braiso4Ea* and *Braiso4Eb* copies were tested (Figure 5B).

A SCAR marker was developed for the *Braiso4Ea* linkage analysis. The primer set was prepared by sequence comparison of the *Braiso4Ea-1* and *a-2* alleles (Figure 3). Primer F1 was designed using the specific insertion sequence of SB18 and SB24 located in intron 1. Primer F2 was based on the specific insertion sequence of SB20 and SB22 located in exon 3. The reverse primer R was derived from the sequence located in intron 3 of *Braiso4Ea-1*, which is shared with the 3′ end of *Braiso4E a-2*. The F1 and R primer set can amplify only *Braiso4Ea-2* in SB18 and SB24 and the expected PCR product size is about 680 bp. The F2 and R primer set can amplify only *Braiso4Ea-1* in SB20, SB22 and GJS2A. Its expected PCR product size is about 190 bp. This was named the trsSCAR marker (Figure 6).

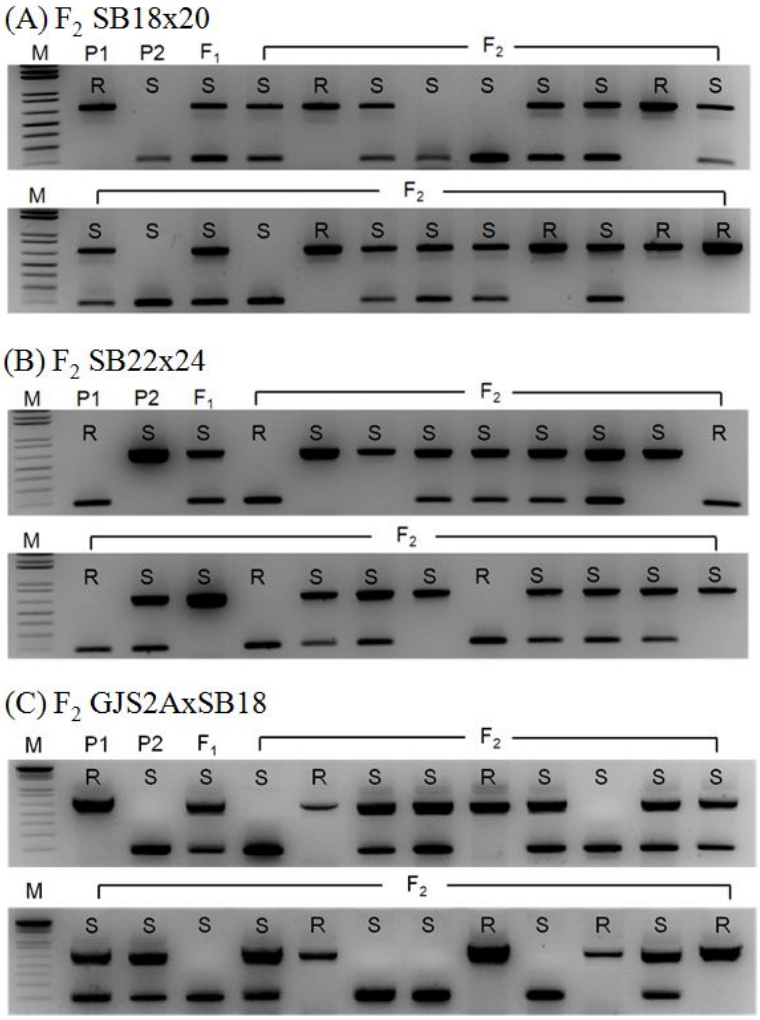


Figure 6. Linkage analysis of trsSCAR marker. (A) Genotype analysis of SB18x20 F_2 using the trsSCAR marker. P1 and P2 refer to SB18 and SB20. (B) Genotype analysis of SB22x24 F_2 using the trsSCAR marker. P1 and P2 refer to SB22 and SB24. (C) Genotype analysis of GJS2AxSB18 F_2 using the trsSCAR marker. P1 and P2 refer to SB18 and GJS2A. M, DNA marker; R, resistant phenotype; S, susceptible phenotype.

To examine if the marker could be used for eIF(iso)4E genotyping of the F_2 population, parental lines, the F_1 plants and F_2 populations of SB18x20, GJS2AxSB18 and SB22x24 were screened with the trsSCAR marker. The band pattern between two resistant (SB18 and SB22), three susceptible parent lines (SB20, SB24 and GJS2A) and their F_1 plants (SB18x20, GJS2AxSB18 and SB22x24) showed polymorphism as expected (Figure 6). Among gDNAs from 71 F_2 population individuals from the SB18x20 cross, 18 F_2 plants showed the band pattern corresponding to the susceptible parental line SB20, 45 plants were confirmed as heterozygous and the other 8 plants showed the same band pattern as the resistant parental line SB18 (Table 3). The gDNAs from 155 GJS2AxSB18 F_2 individuals was also screened with the trsSCAR marker. The band pattern was the same as for SB18x20 F_2. Among these gDNAs, 38 F_2 plants showed the band pattern corresponding to the susceptible parental line GJS2A, 76 plants were confirmed as heterozygous and 41 plants showed the same band pattern as the resistant parental line SB18 (Table 3). Among gDNAs from 57 F_2 population individuals from the SB22x24 cross, 18 F_2 plants showed the same band pattern as the susceptible parental line SB24, 25 plants were confirmed as heterozygous and the other 14 plants showed the same band pattern as the resistant parental line SB22 (Table 3). Plants having the resistant parent genotype showed resistance to TuMV whereas the plants with the

susceptible parent genotype exhibited susceptibility. The ratio among the three genotypes (*TRS/TRS, TRS/trs, trs/trs*) in the F_2 progeny of SB22x24 nearly fitted to 1:2:1 (χ^2 = 1.421, P = 0.491). However, the ratio among the three genotypes in the SB18x20 F_2 progeny was 2.4:5.8:1 and did not fit with the expected 1:2:1 ratio (χ^2 = 7.901, P < 0.05) (Table 3). This may be due to the small F_2 population, which can lead to biased results. Another population, GJS2AxSB18 F_2, showed that the ratio among the three genotypes (*TRS/TRS, TRS/trs, trs/trs*) nearly fitted to 1:2:1 (χ^2 = 0.174, P = 0.917).

Table 3. Co-segregation analysis of TuMV resistance and trsSCAR marker genotype using F_2 populations.

F_2 population	Phenotype	trsSCAR marker genotype		
	S:R	S		R
		TRS/TRS	*TRS/trs*	*trs/trs*
SB18x20	63:8	18	45	8
SB22x24	43:14	18	25	14
GJS2AxSB18	114:41	38	76	41

[a] Resistant plants(R) versus susceptible plants (S)

Linkage analysis of *Braiso4Eb* was also performed. The primers to amplify *Braiso4Eb* were designed using 5′ and 3′ UTR sequences to detect *Braiso4Eb* specifically. A CAPS (Cleaved Amplified Polymorphic Sequences) marker using an XbaI recognition site was developed based on sequence comparison between resistant and susceptible lines (Figure 5A). The susceptible copy has an XbaI restriction enzyme site and is digested into two bands of 1,020 bp and 262 bp (Figure 7). The resistant copy does not have an *Xba*I site. The F_2 population was screened using the marker and the results showed that the F_2 resistance phenotype did not match the band pattern of the susceptible and resistant parents. The same results were obtained for the SB22x24 F_2 population (data not shown). These results indicate that the TuMV resistance in SB18 and SB22 is controlled by the same single recessive gene.

메모 [KJ2]: 수정

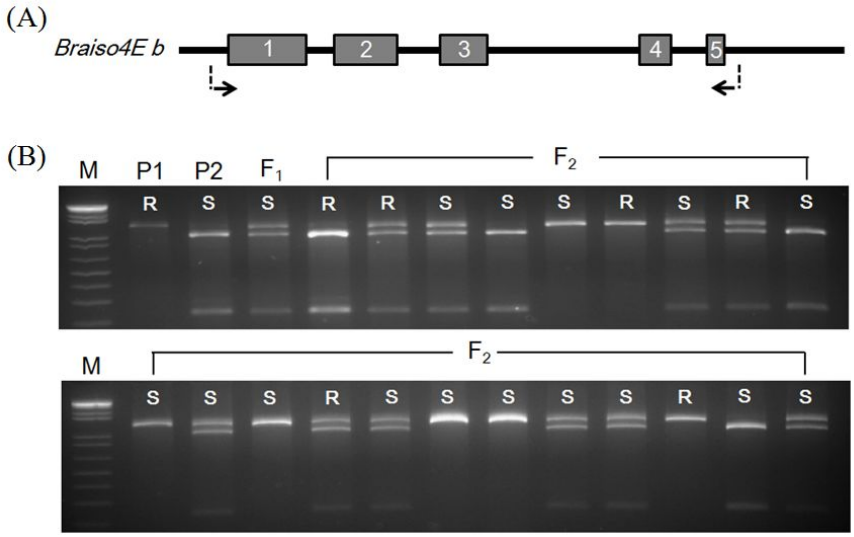


Figure 7. Linkage analysis of *Braiso4Eb*. (A) Primer sites that were used to amplify the *Braiso4Eb* gene copy. (B) Linkage analysis of *Braiso4Eb* using the SB18x20 F_2 population. *Braiso4Eb* was amplified and digested with *Xba1*. P1 and P2 refer to SB18 and SB20. M, DNA marker; R, resistant phenotype; S, susceptible phenotype.

Mapping the *trs* locus

As the sequence analysis of *Braiso4Ea* showed that *Braiso4Ea* is not the gene that controls the TuMV resistance, I searched for other possible candidate genes. The BLAST searches were performed using the Brassica database (BRAD, http://brassicadb.org/brad) (Table 4) to obtain sequences around *Braiso4Ea*. All the homologues of eIF4E family in *Brassica rapa* were isolated previously in the Brassica database (BRAD, http://brassicadb.org/brad). The phylogenetic analysis was conducted using MEGA program version 5 (Tamura et al. 2007) and those homologues were classified into three distinct groups; an eIF4E, eIF(iso)4E, and nCBP (Figure 8). This analysis is based on gDNA data, so there may be pseudogenes that are not expressed. The data analysis showed that multiple copies of eIF4E and eIF(iso)4E exist in *B. rapa*. Among several eIF(iso)4E homologues, *Braiso4Ea* seems to be contained in scaffold 000104 (Bra035393).

Table 4. BLAST and expression analysis of the eIF4E gene family in Brassica.

Arabidopsis	Brassica ID[a]	Chrom	Position of the gene (bp)		Expression[b]	NCBI DB[c]
			Start	End		
eIF(iso)4E	Bra035531	A08	8253220	8254347	+	*BraA.eIF(iso)4E c.*
	Bra035530	A08	8249938	8250288	–	
	Bra039484	A05	9339342	9340622	–	*BraA.eIF(iso)4E b*
	Bra035393	A04	-	-	+	*BraA.eIF(iso)4E a*
eIF4E-1	Bra013283	A01	4729486	4730638	+	*BraA.eIF(iso)4E a*
	Bra012622	A03	23156885	23157543	–	
	Bra021026	A08	10402469	10404041	+	*BraA.eIF(iso)4E c*
eIF4E-3	Bra032325	A09	22556883	22558152	–	
	Bra032326	A09	22551236	22556284	–	
nCBP	Bra002134	A10	11319362	11320645	–	
	Bra006439	A03	3494196	3495491	+	
	Bra023664	A02	4409918	4411407	+	
eIF4E	Bra030147	A07	6233738	6235078	–	

[a] The orthologs found in BLAST searches of the Brassica database (BRAD, http://brassicadb.org/brad), Bra035393 = Scaffold000104

[b] Expression based on representation in the EST database (BrTED, http://brted.rna.kr), + indicates expression and – no expression

[c] The eIF(iso)4E copies found in NCBI blast searches

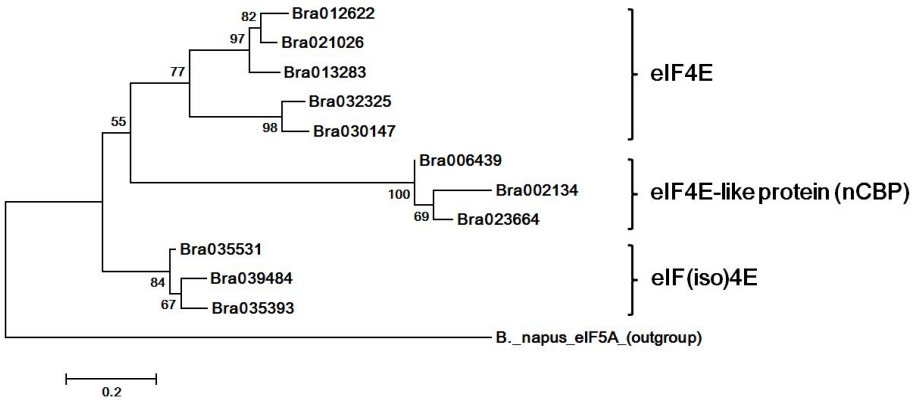


Figure 8. Phylogenetic analysis of eIF4E family of *Brassica rapa* by neighbor-joining algorithm. The analysis was performed using MEGA program version 5 with 1,000 bootstrap replications. All the genes were grouped to three distinct clades as eIF4E, eIF(iso)4E, and nCBP. Minimum evolution, maximum parsimony, and UPGMA algorithms also showed the same the results. The *eIF5A* gene of *B. napus* was used as out-group. Orthologs Bra035530 and Bra032326 were not included because of the drastic size differences.

Preliminary mapping result using VCS40 DH population showed that the *trs* might be located at chromosome A01 or A09 (data not shown). But further efforts to map the precise position of the *trs* gene using A01 or A09 markers showed that none of the developed markers were linked to the *trs* gene indicating that the initial mapping of the *trs* position was wrong. Recently, Qian et al. (2013) reported another single recessive TuMV resistance gene named *retr02*, which is supposed to encode eIF(iso)4E (Qian et al. 2013). According to this study, *retr02* is located on *Brassica* scaffold 000104 on chromosome A04 (Qian et al. 2013). I suspected that the gene *trs* may also be located on chromosome A04, not on chromosome A09 where I previously mapped *trs*.

There are ten scaffolds mapped in chromosome A04 including scaffold 000104 (Qian et al. 2013). To find which scaffold is linked to the *trs* resistance gene, markers were developed from these scaffolds. Developed markers showing clear polymorphism were mapped in two F_2 populations (SB22x24 and GJS2AxSB18). Markers from four scaffolds, scaffold 000104, scaffold 000083, scaffold 040552, and scaffold 000177 showed linkage to the *trs* gene (Figure 9). In the SB22x24 F_2 population, four markers showed linkage to the *trs*. Those are Sca104 10-5 HRM (754.7kb, scaffold 000104), Sca104-1 HRM (543.2kb, scaffold 000104), Sca83 1-2 HRM (56.2kb, scaffold 000083) and Sca552 2-8 HRM (403.2kb, scaffold 040552) (Table 1). All the four

markers perfectly co-segregated with *trs* resistance in the SB22x24 F_2 population. Five markers were mapped in the GJS2AxSB18 F_2 population (Table 1, Figure 9). Among those markers only Sca104 4-2 HRM marker and Sca83 1-7 HRM marker perfectly co-segregated with the *trs* resistance (Figure 9). Sca104-5 HRM marker developed at the lower end of the scaffold 000104 had three recombinants and the Sca552 1-2 HRM marker had one recombinant in the GJS2AxSB18 population (Figure 9). In conclusion, I assumed that the locus of the *trs* is located between Sca104-5 HRM (12.4kb, scaffold 000104) and Sca552 1-2 HRM (28.2kb, scaffold 040552) on chromosome A04 (Figure 9).

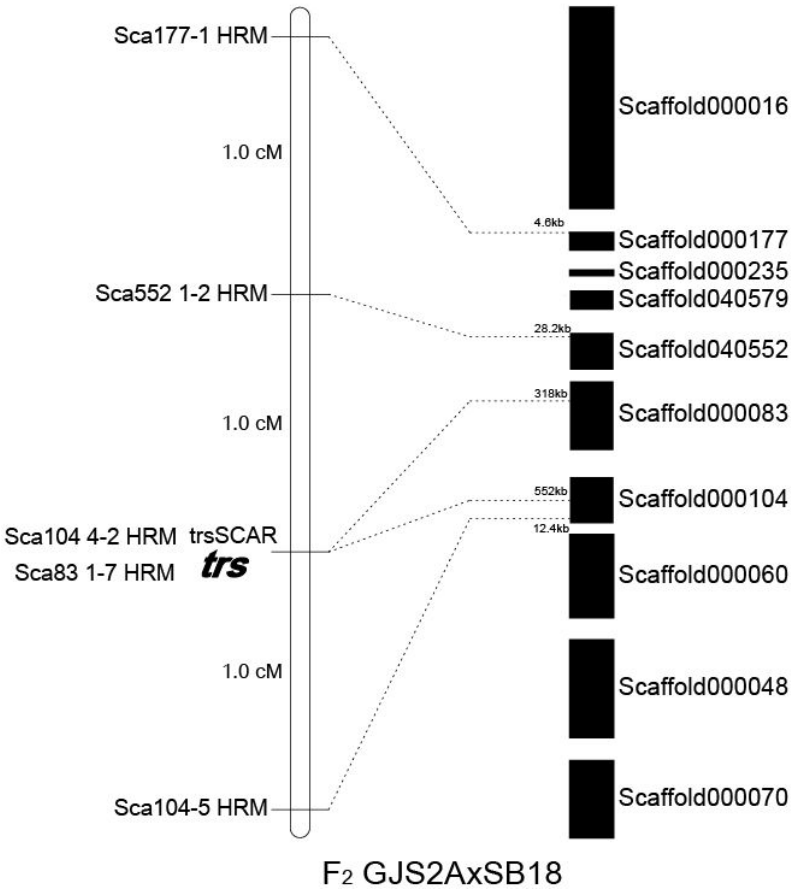


Figure 9. Genetic linkage map of *trs* locus. GJS2AxSB18 F_2 is the mapping population. Genetic distance in cM was calculated using the Kosambi function. The physical map located in the left side is showing the ten scaffolds mapped in chromosome A04 (Qian et al. 2013). The resistance gene, *trs* is located between scaffold000104 and scaffold000083.

Identification of expressed genes of the eIF4E family

To test if the candidate eIF4E/eIF(iso)4E genes are expressed, an EST sequence database was searched for (BrTED, http://brted.rna.kr; Yu et al. 2011) (Table 4). When I looked for the expression of eIF4E gene family members, eIF4E, eIF(iso)4E and nCBP were found to be expressed in *B. rapa*. Fifteen to twenty EST sequences were found for each member (data not shown). Among the eIF(iso)4E members, EST sequences for *Braiso4Ea* and *Braiso4Ec* were identified. However, there was no sequence that showed similarity to *Braiso4Eb*, suggesting that this gene copy is not expressed in *B. rapa*. To confirm this result, 3′ RACE PCR was performed using total RNAs of SB18, SB20, SB22 and SB24. The cDNA sequences of two eIF(iso)4E gene copies, *Braiso4Ea* and *Braiso4Ec,* were amplified and the expression levels between the two gene copies were similar (Figure 10). No products corresponding to *Braiso4Eb* were amplified via 3′ RACE PCR, in agreement with the EST data. No other sequences belonging to the eIF4E gene family other than Bra035393 were found to be expressed in chromosome A04 so far (Table 4).

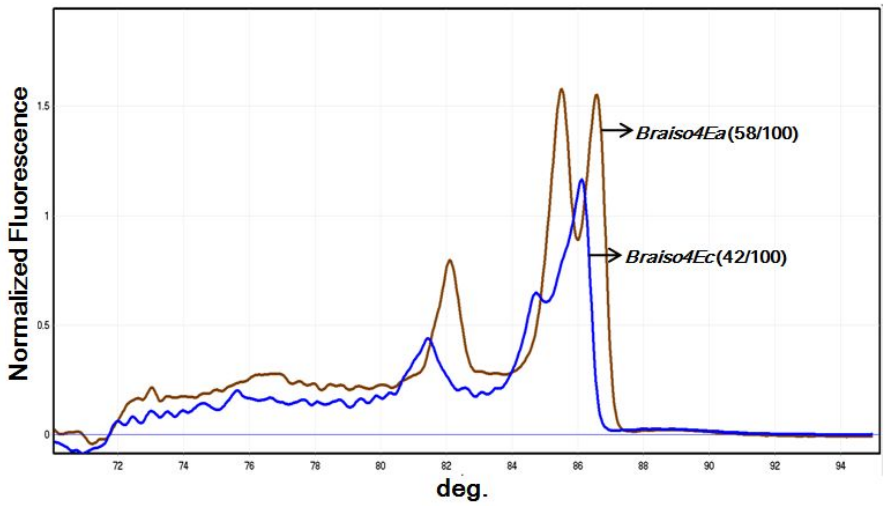


Figure 10. Sorting of colonies containing 3′ RACE product TA clones using HRM. Total colonies were selected as eIF(iso)4E clones. Two melting curves above are showing two copies of eIF(iso)4E, *Braiso4Ea* and *Braiso4Ec.*

DISCUSSION

In this study, a broad spectrum TuMV resistance inherited in a recessive manner was identified. The trsSCAR marker was developed based on two *Braiso4Ea* alleles. Whereas the trsSCAR marker co-segregates with the TuMV resistance, two resistant lines showed opposite genotypes for the marker. This means that *Braiso4Ea* is tightly linked to the TuMV resistance, but that *Braiso4Ea* is not the *trs* gene. Allelism tests showed that the resistance gene in the SB18 and SB22 lines are the same *trs* gene. And the mapping result indicates the *trs* locus is between scaffold 000104 and scaffold 040552 of chromosome A04.

The first recessive resistance gene found in Chinese cabbage was *retr01* (Rusholme et al. 2007). This gene is epistatic to the second dominant gene, *Contro1*, and the locations for these genes are on the upper portions of chromosome 4 and 8 according to the previous research (Rusholme et al. 2007). The dominant and recessive resistance genes, *Contro1* and *retr01* might be related to the eIF4E gene family (Rusholme et al. 2007). But there was no other report showing the actual relationship between the eIF4E family gene and virus resistance in *B. rapa*.

Recently, a single recessive resistance gene *retr02* was identified in

B.rapa (Qian et al. 2013). They speculated that Bra035393 (*BraA. eIF(iso)4E a*) which is located on scaffold 000104, was the *retr02* gene (Qian et al. 2013). The mapping result suggests that the *trs* locus is between scaffold 000104(12.4kb) and scaffold 040552(28.2kb) of the Chromosome A04 (Figure 5). When I aligned the DNA sequence of Bra035393 and *Braiso4Ea-1* (data not shown), *Braiso4Ea-1* sequence showed 100% similarity between that of the Bra035393. According to the research, however, the presence or absence of deletion in the *Braiso4Ea* sequence was not consistent between resistant and susceptible Chinese cabbage lines. Therefore even though *Braiso4Ea* is tightly linked to the *trs*, it is not the *trs* itself. Based on this evidence, I made a conclusion that *retr02* and *trs* gene should be distinguished. But we should still point out that the *trs* locus which was mapped in this study is overlapped with the locus of the *retr02*. The *retr02* was mapped on both scaffold 000060 and scaffold 000104. Then candidate gene approach showed that the *retr02* is on scaffold 000104. The *trs* was mapped between scaffold 000104 and scaffold 040552. Because the identification of the *trs* gene is still on going, it should not be ruled out that *trs* may be another recessive resistance gene tightly linked to allele of the *retr02* or another allele.

So far no other eIF4E family member was found in the chromosome A04. But we still do not know the full sequence of the chromosome A04

because of the gaps between scaffolds due to the constraints of the NGS genome sequencing and abundant repeats in the *B. rapa* genome. So it cannot be ruled out the possibility that *trs* gene may be located in the gap between scaffold000104 and scaffold040552.

As the *Braiso4Ea* was the only copy that showed linkage between *trs,* other candidates for the *trs* gene need to be identified. Other eIF4E or eIF(iso)4E copies may exist besides the sequences I found in this study. Several similar copies of eIF4E and eIF(iso)4E were found in the Brassica database, and these copies are expected to show redundancy to each other. This indicates that eIF4E family proteins may play different roles in different tissues and/or at different developmental stages in a single organism (Duprat et al. 2002; Rhoads et al. 2007). I found a few cases in which eIF4E or eIF(iso)4E sequences are located very near each other in the same chromosome. On chromosome A8, two eIF(iso)4E sequences (Bra 035531 and Bra 031530) are present within a physical distance of about 2930 bp. In chromosome A9, two eIF4E sequences (Bra 032325 and Bra 032326) are close, but they were found to be pseudogenes. Eight copies showing homology with the *Braiso4Ea* were found in the EST database. I am expecting that there may be more homologs of the *Braiso4Ea* copy in Brassica genome that were not identified yet. Because many unfilled gaps in Brassica genome due to the NGS genome sequencing method and

abundant repeats exist. So, *trs* is highly expected to be one of the *Braiso4Ea* homologs. It was also intriguing that EST sequences showing similarity to the *Braiso4Ea* copy tended to have many polymorphisms in their ORF sequences compared to the other copies in the eIF4E gene family. For example, the sequence named EX042260 (256 bp) had six SNPs in its ORF and CV546692 (440 bp) had eleven SNPs. By contrast, *Braiso4Ec* copies and other eIF4E groups showed one to three SNPs on average. eIF4E gene copy sequences appear to be highly conserved compared to eIF(iso)4E gene copies according to these data.

메모 [u3]: 내용 추가

It was challenging to analyze the Brassica sequences because there are several eIF(iso)4E copies. This difficulty in sequence analysis may arise due to the complexity of the Brassica genome. The *B. rapa* genome is organized into ten chromosomes and has emerged as an important model for genomic studies in Brassica species. Comparative studies of Arabidopsis and Brassica prove the event of extensive duplications, with Arabidopsis segments conserved within the diploid Brassica genomes about three times (Lukens et al. 2003; Parkin et al. 2005). The widespread repetitive sequences in the Brassica genome add to the difficulty in analysis. In the case of eIF(iso)4E, there is only a single copy of the eIF(iso)4E gene in Arabidopsis whereas *B. rapa* possesses multiple copies. The confirmed eIF(iso)4E copies that are expressed in *B. rapa* are *BraA.*

eIF(iso)4E a. and *BraA*. *eIF(iso)4E c*. (Jenner et al. 2010).

The *trs* gene confers a high level of resistance to several TuMV strains, CHN2, 3, 4 and 5. Based on previous research, recessive resistance is more durable and provides broad spectrum resistance (Kang et al. 2005; Kang et al. 2007). The barley *mlo* resistance to the powdery mildew fungus is a typical case of broad spectrum recessive resistance (Buschges et al. 1997). *pvr1* from *Capsicum chinense* is also well-known for its broad spectrum recessive resistance to several potyviruses, such as *Potato virus Y* (PVY) pathotypes 0, 1 and 2*, Pepper mottle virus* and most *Tobacco etch virus* (TEV) strains (Kyle and Palloix 1997). *pot-1*, a recessive resistance gene in tomato, mediates resistance to both TEV and PVY (Moury et al. 2004; Parrella et al. 2002). *rym4/5* is also known for controlling the resistance of *Barley yellow mosaic virus* and *Barley mild mosaic virus* (Stein et al. 2005). Several sources of broad spectrum resistance have also been described in *B. rapa* (Hughes et al. 2002; Liu et al. 1996; Rosholme et al. 2007; Suh et al. 1995; Walsh et al. 2002; Yoon et al. 1993). It seems that broad spectrum resistance in Brassica tends to be controlled by recessive genes but the genetic characterization of the recessive resistance was unclear in many cases. *B. rapa* resistance line 0-2 is effective against five TuMV strains CHN1–5 and was reported to be controlled by two recessive genes when tested by strains CHN4 and 5 (Yoon et al. 1993). Another

resistance line RLR22 contains two resistance genes, the recessive gene *retr01* and the dominant gene *Contr01*. It also exhibited broad spectrum resistance against eight different TuMV strains (Rusholme et al. 2007).

The *trs* gene was inherited as a single recessive gene according to the research. But unlike that of the SB22x24 population, the genetic ratio of the SB18x20 population did not fit to an expected Mendelian ratio. The ratio was approximately 1:8 (resistant versus susceptible) in these data. This result can be ascribed to the small size of the F_2 population. So I tested another SB18 derived F_2 population (GJS2AxSB18) which size was increased more than twice. This F_2 population showed an expected ratio of 1:3 (resistance versus susceptible).

The symptoms of the F_1 Chinese cabbage lines were different from those of the susceptible parent although virus coat protein accumulation was similar. The virus symptoms of the F_1 plants were much weaker than those of the susceptible parents. The same trend was observed in homozygous resistant compared to heterozygous susceptible F_2 individuals. The symptoms caused by viral systemic infection vary due to the differences in host factor-virus interactions (Kaneko et al. 2004; Kim et al. 2010). Therefore, even though host proteins are controlled by recessive alleles, it appears that the differences in host proteins produced in homozygous and heterozygous plants affect virus symptom development. Previous studies on over-expression of recessive

resistance alleles in tomato and potato systems support this idea. The ectopic expression of mutated eIF4E gene of pepper induced strong resistance to multiple viral species in the tomato system (Kang et al. 2007). Another recent report also showed that transgenic expression of the *pvr1*^{2} gene from pepper confers resistance to PVY in potato (Cavarorta et al. 2011). Over-expressed eIF(iso)4E mutant protein overwhelmed the normal eIF(iso)4E and the virus-host interaction was disturbed. These reports are consistent with my observation of weak symptoms in the heterozygous plants.

The broad spectrum resistance introduced in this study represents a novel and potentially durable source of resistance to TuMV. Because this resistance is extremely strong and is controlled by only a single gene, this TuMV resistance source may be useful in developing resistant cultivars especially in Korea and China, where the damage caused by TuMV is severe. The newly developed SCAR markers can be used in marker-assisted selection for TuMV resistance. Because the SCAR marker I developed is based on the copy linked to the *trs* gene, the *trs* gene itself should be identified in order to utilize the resistance more efficiently. Furthermore, identification of the *trs* gene will elucidate fundamental mechanisms of the host-virus interaction in *B. rapa* and contribute to better understanding of virus resistance in plants.

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CHAPTER II

Engineering TuMV Resistance Transgenic Plants Using eIF(iso)4E in *Brassica rapa*

ABSTRACT

Turnip mosaic virus (TuMV), which belongs to the genus *Potyvirus*, is one of the major viruses in Brassicaceous plants. It is known that the interaction between TuMV VPg and eIF4(iso)E is a critical step in determining the virulence. To identify the key amino acids in the interaction between TuMV VPg and Brassica eIF(iso)4E, amino acids of eIF(iso)4E in the cap-binding pocket were mutated. Seven amino acids in the cap-binding pocket were selected for candidate amino acids that may play a role in the interaction with TuMV VPg. Using a yeast two-hybrid system, it was demonstrated that a single amino acid mutation in cap-binding pocket of Brassica eIF(iso)4E could

interrupt the interaction with TuMV VPg. eIF(iso)4E which has a mutation at each of the amino acid position, W49, W95 and K150, was impaired significantly in its interaction with the VPg. A co-immunoprecipitation analysis also supports the result. Complementation of an eIF4E-knockout yeast strain by the mutated eIF(iso)4E genes showed that all eIF(iso)4E mutants were able to complement eIF4E of the yeast, indicating the mutated eIF(iso)4E retained their function. To determine if these mutations are able to confer resistance to the Chinese cabbage, transformants over-expressing eIF(iso)4E mutants were developed. eIF(iso)4E W95L, W95L/K150E and eIF(iso)4E wild-type were over-expressed in a susceptible Chinese cabbage cultivar. According to the TuMV screening result of T_1 and T_2 transformants, over-expression of the eIF(iso)4E mutants showed resistance to all four TuMV strains (CHN2, 3, 4 and 5). These results support the hypothesis that mutations in eIF(iso)4E can engineer broad-spectrum TuMV resistance in Chinese cabbage.

INTRODUCTION

Turnip mosaic virus (TuMV) belongs to the *Potyvirus* genus. The viral genome of TuMV is a positive-sense single-stranded RNA molecule of about 10 kb in length. The virus has a poly (A) tail at the 3′-end, and a VPg (22–24 kDa) covalently linked to the 5′-end. This protein is well-known for the interaction with eukaryotic translation factors such as eIF4E. In eukaryotic cells, most of the nuclear encoded mRNA has an m^7GTP cap structure in the 5′-end. The eukaryotic initiation factors 4E (eIF4E) interacts with the mRNA by binding to the 5′cap and associates with eIF4G (Tarun and Sachs, 1996). Plants have a second form of eIF4E and eIF4G, named eIF(iso)4E and eIF(iso)4G (eIF(iso)4F) respectively (Browning et al. 1987; Bush et al. 2009).

All potyvirus recessive resistance genes that were identified so far are known to encode initiation factors such as eIF4E and eIF4G (Robaglia and Caranta. 2006; Wang et al. 2012). For example, *pvr1, pvr2 and pvr6* in pepper (Kang et al. 2005b; Ruffel et al. 2006; Charron et al. 2008), *mo1* in lettuce (Nicaise et al. 2003), *nsv* in melon (Nieto et al. 2006), *sbm1* in pea (Gao et al. 2004b), *pot-1* in tomato (Ruffel et al. 2005), *lsp1*, *cum1* and *cum2* resistance

alleles created by mutagenesis in *Arabidopsis thaliana* (Lellis et al. 2002; Yoshii et al. 2004), *wlv* and *cyv2* in pea (Bruun-Rasmussen et al. 2007; Andrade et al. 2009), *tsv1* and *rymv-1* in rice (Albar et al. 2006; Lee et al. 2010) and *bc-3* in bean (Naderpour et al. 2010). *rym4, rym5 and rym6* in barley which control resistance toward bymoviruses were also identified as *eIF4E* gene (Kanyuka et al. 2005; Stein et al. 2005). In Chinese cabbage, *retr01, retr02* and *trs* are supposed to be related to the eIF4E gene family (Rusholme et al. 2007; Qian et al. 2013, Kim et al. 2013). Mostly these resistance genes confer extreme resistance (Provvidenti and Hampton. 1992). These eIF4E or eIF(iso)4E mediated resistances are determined by a few amino acid mutations in the protein. Though the mechanism of this resistance is not clear, the interaction of eIF4E and the potyvirus VPg very often seems to be related in this resistance.

VPg is a multifunctional protein which is predicted to be involved in various part of virus infection cycle including replication, translation, proteolysis, cell-to-cell movement, RNA synthesis and an inhibitor of a plant antiviral agent (Siaw et al. 1985; Riechmann et al. 1989; Murphy et al. 1990; Riechmann et al. 1990; Revers et al. 1999; Puustinen et al. 2002; Anindya et al. 2005; Domashevskiy et al. 2012). VPg acts as an avirulence factor of many plants (Moury et al. 2004; Kang et al. 2005a; Bruun-Rasmussen et al. 2007; Perez et al. 2012). Many researchers regard VPg as the central protein of the

huge interactome between host and virus proteins (Jiang and Laliberte. 2011). Among the diverse interactions, eIF4E- or eIF(iso)4E-VPg is the most wildly studied. Several researches have shown the physical interactions between potyvirus VPg or VPg Pro and eIF4E or eIF(iso)4E via various methods (Wittmann et al. 1997; Leonard et al. 2000; Schaad et al. 2000; Leonard et al. 2002; Yeam et al. 2007; Charron et al. 2008, Khan et al. 2008). It has been suggested that these interactions are important in viral RNA translation and/or replication (Wittmann et al. 1997; Leonard et al. 2000; Grzela et al. 2006; Miyoshi et al. 2006; Robaglia and Caranta. 2006; Beauchemin and Laliberte. 2007; Cotton et al. 2009). Many viral resistance genes encoding members of the eIF4E family are an altered form of the susceptible gene which affect the interaction with VPg (Ruffel et al. 2002; Nicaise et al. 2003; Gao et al. 2004b; Kang et al. 2005a; Ruffel et al. 2005; Yeam et al. 2007). Hence, the amino acid substitution in the translation initiation factor may impair virus replication and translation. The previous reports about the VPg binding site suggest that VPg may bind at or near the cap-binding pocket of the eIF4E protein family (Leonard et al. 2000; Miyoshi et al. 2006). Most of the substitutions of eIF4E/eIF(iso)4E that confer viral resistance were located at or near the cap binding pocket (Robaglia and Caranta. 2006; Charron et al. 2008; Truniger and Aranda. 2009).

The eIF4E-based resistance was deployed for decades in many crops and it appears that it is difficult for plant viruses to mutate easily to compensate for its interaction with eIF4E, probable due to the fitness penalty (Kang et al. 2005b). This may be the reason that the eIF4E-based recessive resistance is regarded as more durable than R gene-mediated resistance. Although it is known that the eIF4E mediated resistance is a successful way to manage virus diseases, such resistance genes are not always found in nature. Using biotechnology to engineer novel resistance alleles can be an alternative approach at this time. The advantage of engineering resistant plants is that it can preserve other necessary traits with the target allele (Cavatorta et al. 2011). In these days, there are two ways in engineering resistant plant using eIF4E. One way is to use TILLING (Targeting Induced Local Lesions IN Genomes) technique in screening the mutant population derived by EMS or other mutagens. It helps to select the target-gene-mutated plants quickly. The other way is to develop transgenic plant by silencing or over-expressing the specific protein which is related to resistance. Many studies suggest that absence of the wild-type (w/t) host factor or over-expression of the mutated host factor affects the virus susceptibility (Lellis et al. 2002; Yoshii et al. 2004; Ruffel et al. 2005; Kang et al. 2007; Yeam et al. 2007; Piron et al. 2010; Cavatorta et al. 2011; Mazier et al. 2011; Rodriguez-Hernandez et al. 2012; Contreras-Paredes et al.

2013; Wang et al. 2013). Kang et al. (2007), Cavatorta et al. (2011) and Duan et al. (2012) especially showed an effective strategy to obtain virus resistance via over-expression of a mutated eIF4E gene.

TuMV has a broad host range but the major host of the virus is Brassicaceous plants. Drastic economic losses in several vegetable crops are due to TuMV (Edwardson and Christie. 1991; Shattuck et al. 1992). Serious economic loss of Brassica crops including Chinese cabbage by TuMV arise every year especially in China. There was a report suggesting that TuMV might use both Brassica eIF4E and eIF(iso)4E proteins in *A. thaliana* (Jenner et al. 2010). In other reports, eIF(iso)4E of *A. thaliana* turned out to be a key factor in the TuMV resistance mechanism (Leonard et al. 2000; Lellis et al. 2002; Duprat et al. 2002; Beauchemin et al. 2007; Miyoshi et al. 2008). Recent research showed the Brassica eIF(iso)4E gene is highly related to the Brassica recessive resistance genes (Rusholme et al. 2007; Qian et al. 2013; Kim et al. 2013).

The purpose of this study is to engineer a broad-spectrum resistant Chinese cabbage by using the mutated eIF(iso)4E genes. In 2006, a three dimensional model of the *A. thaliana* eIF(iso)4E was predicted and several amino acids that might be located in the cap binding pocket were reported (Miyoshi et al. 2006). The target amino acids were chosen based on the Arabidopsis cap-binding pocket and the amino acids were mutated. The yeast

two-hybrid and co-immunoprecipitation analysis identified that some amino acids among the candidates may be critical in the TuMV VPg interaction. The over-expression study of mutated eIF(iso)4E in susceptible Chinese cabbage resulted in transgenic plants having broad-spectrum TuMV resistance.

MATERIALS AND METHODS

Plant and virus materials

Brassica rapa subsp. Pekinensis 'Samjin' which is the cultivar developed by Monsanto Korea (Seoul, Korea) was used as a susceptible control and was used for the cloning of susceptible eIF(iso)4E cDNA. Another *Brassica rapa* cultivar 'Seoul' was used for the transformation of eIF(iso)4E. The plant sample of TuMV natural resistant Chinese cabbage was also provided by the National Institute of Horticultural and Herbal Science. TuMV CHN2, 3, 4 and 5 were provided by Namhan Huh (Nongwoo Bio, Yeojoo, Korea). Virus inoculum was propagated in Chinese cabbage 'Samjin' (Monsanto Korea, Chochiwon, Korea).

RT-PCR and TA cloning

Total RNA was isolated from transformed Chinese cabbage using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen Life Technologies) and the oligo (dT). Full length eIF(iso)4E cDNA sequences were amplified with gene-specific primers (Table 1). The PCR was performed in 50 μl reaction volumes with 50-100 ng of DNA as template, 1 x PCR buffer (Takara Shuzo Co., Kyoto, Japan), 2.5 mM dNTP, 1.25 units of EX-Taq (Takara

Shuzo Co., Kyoto, Japan) and 5 pmol of each primer. The PCR conditions were 94°C for 30 s, 55 °C for 30 s, 72 °C for 1 min, for 30 cycles. DNA fragments were cut from the gel and DNA was recovered using a Zymo Gel Recovery kit (Zymo Research, Orange, CA, USA). PCR products were cloned using a TOPO TA Cloning Kit (with pCR2.1-TOPO vector, Invitrogen Life Technologies, Carlsbad, CA, USA). The transgene sequences were confirmed at NICEM (Seoul National University, Seoul, Korea) by the Sanger method.

PCR procedure

PCR reactions to amplify cDNAs were done in 25 ul with 2.5 μl of 10×EX-Taq polymerase buffer (Takara Shuzo Co., Kyoto, Japan), 2μl of 2.5 mM deoxyribonucleotide triphosphate, 2.5 units of EX-Taq (Takara Shuzo Co., Kyoto, Japan), 5 pmol of each primer. The PCR condition was 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, for 17 cycles. Primers that were used for cDNA synthesis of eIF(iso)4E and TuMV VPg are listed below (Table 1).

Table 1. Primer sequence used in plasmid construction and eIF(iso)4E site directed mutagenesis.

Primer ID	Primer sequence	Direction
TuMV VPg (*Nco1*)	CCATGGATGGCGAAAGGCAAGAGGCA	F
TuMV VPg (*Sal1*)	GTCGACCTATCGTGGTCCACTGGGAC	R
eIF(iso)4E (*EcoR1*)	GAATTCATGGCGACAGAGGATGTGAA	F
eIF(iso)4E (*Xho1*)	CTCGAGTCAGACAGTGAACCGAGTTC	R
W49L	CAAGGCGCCGCCCTTGGAGCCTCCCTTCG	F
	CGAAGGGAGGCTCCAAGGGCGGCGCCTTG	R
W95L	GTTGAGCCTAAGCTTGAAGATCCTGAG	F
	CTCAGGATCTTCAAGCTTAGGCTCAAC	R
K105L	GCTAATGGCGGACTTTGGACTTTTGTT	F
	AACAAAAGTCCAAAGTCCGCCATTAGC	R
T107L	GGCGGAAAGTGGCTTTTTGTTGTTACC	F
	GGTAACAACAAAAAGCCACTTTCCGCC	R
K150L	AGTGTGCGCCCACTTCAGGACAAGCTC	F
	GAGCTTGTCCTGAAGTGGGCGCACACT	R
D191L	ACTAACCATGATCTTTCTAGAAGAACT	F
	AGTTCTTCTAGAAAGATCATGGTTAGT	R
S192L	AACCATGATGATCTTAGAAGAACTCGG	F
	CCGAGTTCTTCTAAGATCATCATGGTT	R
RT PCR eIF#1	CTTCTGGGGTTTGCACGA	F
	CCGACAAGAGCCATCAAAGTTTC	R
HPT2	CCTGAACTCACCGCGACG	R
	AAGACCAATGCGGAGCATAT	F
Br actin F	GTGACAATGGAACTGGAATGG	F
	AGACGGAGGATAGCGTGAGG	R

Virus screening procedure

Plants were inoculated at the three-to-four-leaf stages by mechanical inoculation. Before the inoculation, virus inoculum was prepared by grounding TuMV inoculated plant leaves in 50 mM potassium phosphate buffer (pH 7.5). Mechanical inoculation was carried out by applying virus inoculum with light Carborundum dusting. After 20 dpi (days post inoculation), plants were screened daily. The resistance and susceptibility was determined by the absence or presence of visual symptoms. The data was confirmed again by DAS-ELISA. The ELISA antibodies used were obtained by Kisan Biotech Co. Ltd, Seoul, Korea. When the ELISA absorbance value was higher than 2.5 times the mean absorbance value of three un-inoculated samples, the plants were classified as susceptible.

Site-directed mutagenesis and plasmid constructs

eIF(iso)4E coding sequence from Chinese cabbage 'Samjin' was cloned into the vector pJG4-5. eIF(iso)4E mutagenesis was carried out as described (Simionatto et al. 2009) with some modifications. The coding regions of all eight produced mutants were cloned using TOPO TA Cloning Kit with pCR2.1-TOPO vector) (Invitrogen Life Technologies, Carlsbad, CA, USA) and then subcloned into the pJG4-5 vector. The eIF(iso)4E forward primer 5′-

GAATTCATGGCGACAGAGGATGTGAA-3′ and the eIF(iso)4E reverse primer 5′-CTCGAGTCAGACAGTGAACCGAGTTC-3′ containing restriction sites for cloning in the pJG-4-5 vector were designed. Primers that were used in site- directed mutagenesis are listed in Table 1.

Yeast two-hybrid analysis

Yeast two-hybrid analysis using the yeast strain EGY48 and vectors pEG202, pJG4-5 and pSH18-34 were carried out as described (Kang et al. 2005a; Yeam et al. 2007). A bait plasmid, pEG202, was used for the fusion of TuMV VPg and a prey plasmid, pJG4-5, was used to express eIF(iso)4E from Chinese cabbage containing each substitution separately. Because the lacZ reporter plasmid pSH18-34 is present in the yeast cells, interaction of the proteins were measured by a β-galactosidase assay. Yeast transformants were streaked to minimal medium agar plates containing 40 μg/ml X-gal to assay expression of the lacZ reporter gene. The quantitative assay for determining β-galactosidase activity using CPRG was performed as described in the manufacturer's protocol (CLONTECH).

Bimolecular fluorescence complementation assay

The eIF(iso)4E and TuMV VPg cDNAs were cloned into pSPY-CE and NE, respectively. For the *Nicotiana benthamiana* infiltration, the *Agrobacterium tumefaciens* strain GV2260 was infiltrated into the abaxial air space of 3 week-old. The p19 protein of *Tomato bushy stunt virus* was used to suppress gene silencing. Epidermal cell layers of tobacco leaves were assayed for fluorescence 2 days after infiltration. All images are projection stacks of multiple confocal sections produced with a Leica LCS-SL CLSM. Bars represent 50μm.

Co-immunoprecipitation analysis

Purified plasmid DNA of each pEG201-HA and pEG202-FLAG constructs were electroporated into *Agrobacterium tumefaciens* GV2260 (Earley et al., 2006). HA-TuMV VPg and FLAG-eIF(iso)4E (wild type and mutants) were co-expressed *in N. benthamiana* by Agrobacterium-mediated transient expression. HA-GFP and FLAG-GFP were used as negative control. Two leaves were infiltrated with agrobacterium and were collected 72 hr after the infiltration. The condition of extraction buffer was GTEN buffer, 0.1% Triton X-100, 10mM DTT (Sigma Aldrich), 2% PVPP (Sigma Aldrich), 0.5% plant protein protease inhibitor cocktail (Sigma Aldrich) and 0.5% protein

phosphatase inhibitor cocktail (Sigma Aldrich). IP buffer that was used for the immunoprecipitation reaction was consisted with GTEN buffer, 0.15% Nonidet P-40 and 2 mM DTT. Co-immunoprecipitation was performed by previously described protocol (Oh and Martin. 2011). Protein extracts were incubated for 4 hours at 4°C with anti-HA agarose conjugated beads (Sigma Aldrich, St. Louis, Missouri, USA). The collected beads were washed eight times with the IP buffer. The immunoprecipitants were washed and then boiled for 5 min in one volume of 5X sample buffer containing 1M Tris-HCl (pH6.8), 10% SDS, 50% glycerol, 5% β-mercaptoethanol and 1% bromophenol blue. The proteins were separated via 15% SDS-PAGE acrylamide gels and immunoblotted with anti-HA antibody (Sigma) and anti-FLAG antibody (Sigma).

Plant transformation analysis

eIF(iso)4E cDNA acquired by RT-PCR was cloned into the plant transformation vector pMDC32, which contains a *Cauliflower mosaic virus* 35S promoter. eIF(iso)4E w/t, W95L single mutants and W95l/K150E double mutant were cloned for the transformation. Purified plasmid DNA of each pMDC32 construct see below (Figure 3A) was electroporated into *A. tumefaciens* LBA4404. The presence and stability of the transgenes was verified by PCR. Chinese cabbage cultivar 'Seoul' was transformed using leaf

disk method at National Institute of Horticultural and Herbal Science. For the T_1 selection, hygromycin (15mg/mL) containing ½ Murashige and Skoog medium was used. The ploidy of the transformants was also confirmed at the National Institute of Horticultural and Herbal Science (Table 2).

Table 2. eIF(iso)4E over-expressing T_1 plants and copy number of the transgene.

eIF(iso)4E transgene	**T_1 Plant ID**	**Ploidy**
Wild-type	K1-2	4
	K1-3	2
	K1-4	2
	K1-5	2
	K1-6	2
W95L single mutant	K3-1	2
	K3-2	4
	K3-3	4
	K3-4	4
	K3-5	4
	K3-6	4
	K3-7	4
	K3-8	2
	K3-9	2
	K3-11	4
	K3-13	2
	K3-14	4
	K3-16	2
	K3-17	2
W95L/K150E double mutant	K2-1	2
	K2-2	2
	K2-3	4

Yeast complementation analysis

Saccharomyces cerevisiae strain J055 (cdc33-: LEU2 Leu2 ura3 his3 trp1 ade2 [YCp33supex-h4E URA3]) containing a deletion of the chromosomal gene coding for eIF4E was kindly provided by Dr. Carole Caranta (INRA, France). The survival of the yeast depends on the presence of plasmid YCp33supex-h4E URA3 containing a copy of the human eIF4E cDNA, under the control of the glucose-repressible, galactose-dependent GAL promoter. The Chinese cabbage coding sequences of each w/t and mutant *eIF(iso)4E* alleles were cloned into the p424GBP/TRP1 glucose-dependent vector and independently used to transform *S. cerevisiae* strain JO55. After transformation, yeast cells were grown in appropriate selective nutrient drop-out media containing 2% glucose and tested at 30^{o}C for their ability to complement the lack of endogenous eIF4E.

RESULTS

Site-directed mutagenesis of cap-binding pocket amino acids in eIF(iso)4E from Chinese cabbage

It is assumed that expected that key amino acid residues in Arabidopsis cap-binding pocket would be conserved among other plant that belongs to the Brassicaceae family. And on the basis of the VPg-binding model, there may be a key amino acid that is highly necessary in VPg interaction among the amino acids in cap-binding pocket. Based on a previous study (Miyoshi et al. 2006) describing the cap-binding pocket of Brassica eIF(iso)4E, seven candidate amino acids (Trp49, Trp95, Lys105, Lys150, Thr107, Ser192, and Asp191) were chosen (Figure 1). To determine their importance in eIF(iso)4E-VPg interaction, site-directed mutagenesis was performed. These candidate amino acids were successfully mutated to leucine by PCR-based, site -directed mutagenesis. Seven single amino acid mutated eIF(iso)4Es (W49L, W95L, K105L, K150L, T107L, S192L and D191L) and one double amino acids mutated eIF(iso)4E (W95L/K150E) were prepared for further analysis.

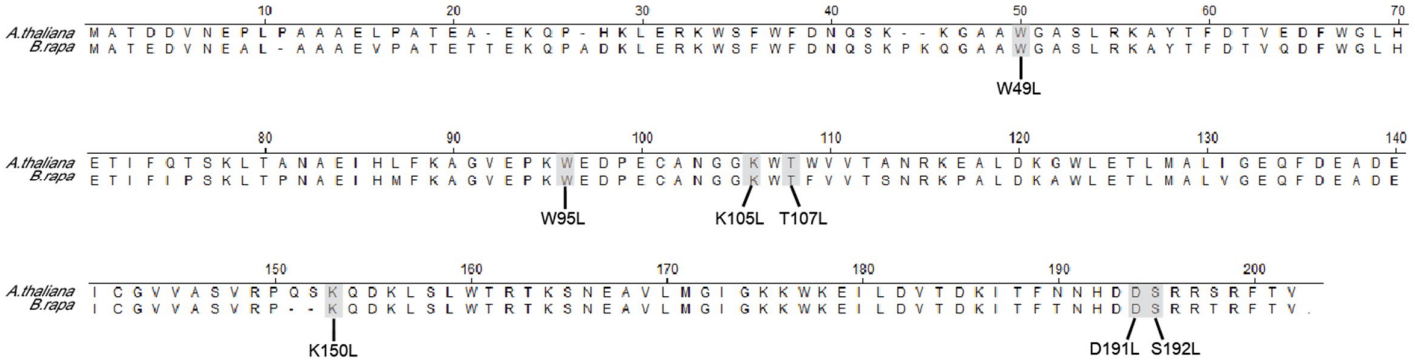


Figure 1. The position of the mutated amino acids in cap binding pocket of eIF(iso)4E.
A. thaliana and *B. rapa* eIF(iso)4E amino acids were aligned. Seven candidate cap binding pocket amino acids (Trp49, Trp95, Lys105, Thr107, Lys150, Asp191 and Ser192) from *B. rapa* that were mutated are indicated as grey boxes in the sequence alignment.

***In vitro* interaction of eIF(iso)4E and TuMV VPg in the yeast two-hybrid system**

To analyze the effect of each amino acid mutation on the eIF(iso)4E-VPg physical interaction, a yeast two-hybrid system was used to measure their interaction. Eight eIF(iso)4E mutants were transformed into yeast strain EGY48. The eIF(iso)4E from 'Samjin' cultivar was also transformed in the yeast as for a positive control and empty vectors were transformed as a negative control. Qualitative β-galactosidase assays showed that the double mutated eIF(iso)4E W95L/K150E reduced its interaction remarkably with TuMV VPg compared to that of the positive control (Figure 2A). eIF(iso)4E mutants that had single mutation in W49, W95, K150 and S192 also showed very weak interactions with VPg as did the W95L/K150E mutant (Figure 2A). On the other hand, the K105L, T107L and D191L eIF(iso)4E mutants showed strong interactions with VPg (Figure 2A). Quantitative β-galactosidase assays (CPRG liquid assay) confirmed the result of qualitative β-galactosidase assays. The β-galactosidase activity of the mutant eIF(iso)4E W95L was similar to the negative control (Figure 2B). The W95L/K150E, W49L and K150L mutants also showed very low β-galactosidase activity, although these activities were slightly higher than

that of the negative control (Figure 2B). The β-galactosidase activities of the K105L, T107L and D191L eIF(iso)4E mutants were even higher than that of the positive control (Figure 2B). In summary, yeast two-hybrid analysis showed that single mutation at W49, W95, K150 and S192 amino acid residues induces the loss of VPg interaction.

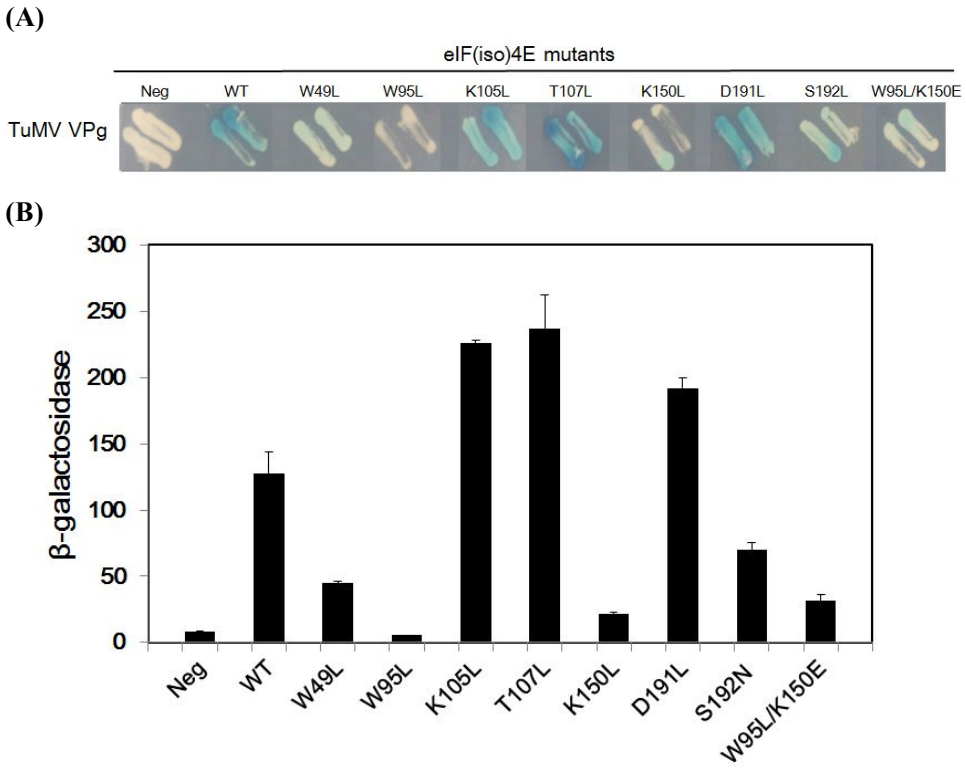


Figure 2. *In vitro* interaction between TuMV VPg and Chinese cabbage eIF(iso)4E. Bait plasmid pEG202 was used to express the fusion protein VPg from TuMV while the prey plasmid pJG4-5 was used to express wild type and mutated eIF(iso)4Es. (A) Yeast transformants were streaked to minimal medium agar plates containing 40 μg/ml X-gal to assay expression of the *lacZ* reporter gene, indicated by a developing blue color. (B) Quantitative liquid culture assay using CPRG as substrate was used to calculate β-galactosidase activity. One unit of β-galactosidase equals the amount that hydrolyzes 1 μmol of CPRG to chlorophenol red and D-galactose per minute per cell (P-value≥0.05). Neg, negative control; WT, susceptible wild type Samjin, W49L, eIF(iso)4E W49L mutant; W95L, eIF(iso)4E W95L mutant; K105L, eIF(iso)4E K105L mutant; T107L, eIF(iso)4E T107L mutant; K150L, eIF(iso)4E K150L mutant; D191L, eIF(iso)4E D191L mutant; S192L, eIF(iso)4E S192L mutant; W95L/K150E eIF(iso)4E W95L/K150E double mutant.

***In vivo* interaction of eIF(iso)4E and TuMV VPg in the Bimolecular fluorescence complementation assay**

To investigate the interaction between TuMV VPg and the eIF(iso)4E mutants *in planta*, a BiFC assay was carried out. Arabidopsis bZIP protein, a transcription factor which is known to form homodimers and heterodimers via the C-terminal leucine zipper domain (Siberil et al. 2001), was used for the positive control based on the previous research (Walter et al. 2004), and the Green Fluorescent Protein (GFP) was used as a negative control. The TuMV VPg was fused to the N-terminal fragment of Yellow Fluorescent Protein (YFP) (TuMV VPg-YN) and co-expressed with the fusion of eIF(iso)4E to the C-terminal fragment of YFP (eIF(iso)4E-YC). The green signal in the figure represents the BiFC signal which indicates the interaction between two proteins. The interaction between eIF(iso)4E w/t and TuMV VPg was confirmed when these proteins were co-expressed (Figure 3A). The YFP fluorescence was distributed throughout the cytoplasm and the nucleus but the fluorescence signal was weaker than expected (Figure 3A). There were no signals apparent in the two eIF(iso)4E mutants (W95L and W95L/K150E) which were indistinguishable from the negative control (Figure 3A), but the co-expression of eIF(iso)4E K150L and TuMV VPg showed a faint fluorescence signal

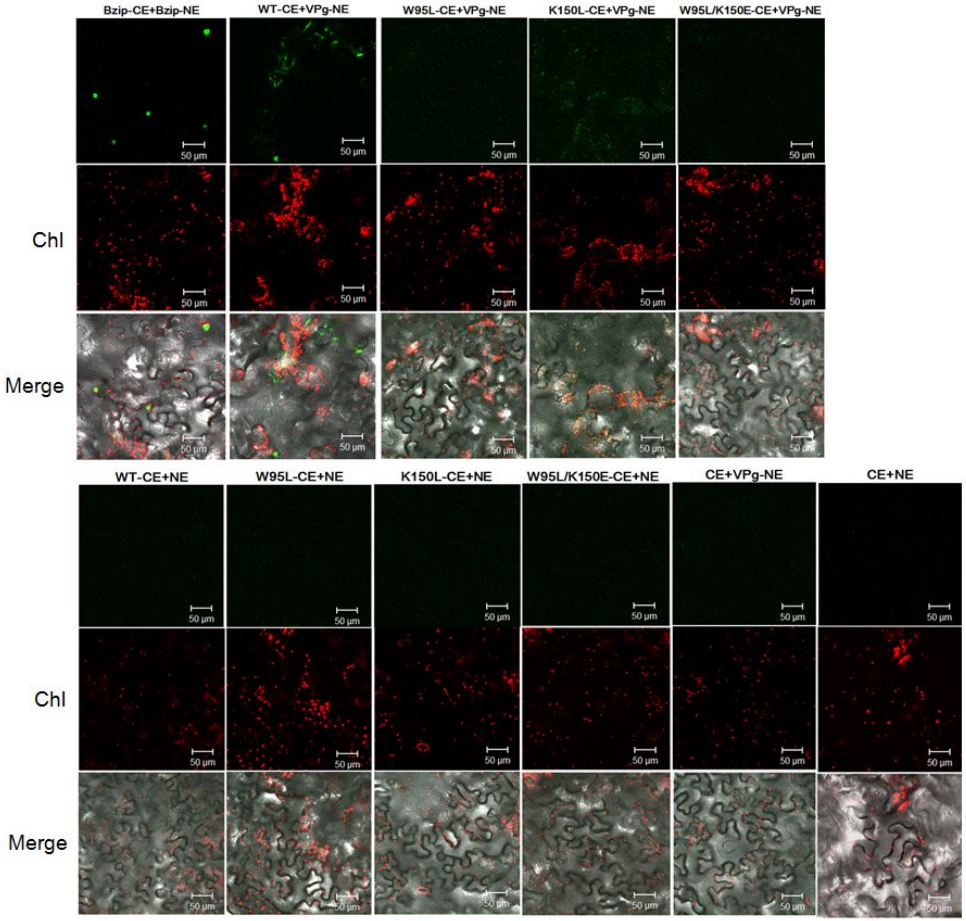
(Figure 3A). It seems that this eIF(iso)4E mutation does not abolish fully the VPg interaction. Due to the weak interaction signal of the eIF(iso)4E w/t and TuMV VPg, it was difficult to conclude that these mutants are actually disturbing the eIF(iso)4E-TuMV VPg interaction *in vivo* as in the case of the *in vitro* study.

Because it was not available to explain yeast two-hybrid data in plant by BiFC, co-immunoprecipitation using HA tag and FLAG tag was performed (Figure 3B). HA-tagged TuMV VPg was co-expressed with FLAG-tagged eIF(iso)4Es. Total protein extracts were immunoprecipitated with anti-HA agarose beads and immunoblotted. The uniformed expression of TuMV VPg was confirmed among all the samples after immunoprecipitation. All FLAG-tagged eIF(iso)4Es were all co-immunoprecipitated with HA-TuMV VPg. But there was a difference between eIF(iso)4E w/t and three mutants. The direct interaction of eIF(iso)4E w/t and VPg was detected as expected while W95L/K150E and K150L mutants showed clear reduction of eIF(iso)4E-TuMV VPg interaction, as indicated by co-IP result. It seems that the interaction of W95L was also slightly reduced compared to the w/t, but not as much as the other two mutants. All the FLAG-tagged eIF(iso)4Es did not co-immunoprecipitated with the negative control, GFP-HA.

The result indicates that there is a clear interaction between Brassica

eIF(iso)4E wild type and TuMV VPg (Figure 3B). It also suggests that the mutations in these targeted amino acids are effective in reducing the interaction efficiency of VPg (Figure 3B).

(A)


Bzip-CE+Bzip-NE
WT-CE+VPg-NE
W95L-CE+VPg-NE
K150L-CE+VPg-NE
W95L/K150E-CE+VPg-NE
Chl
Merge
WT-CE+NE
W95L-CE+NE
K150L-CE+NE
W95L/K150E-CE+NE
CE+VPg-NE
CE+NE
Chl
Merge
50 μm

(B)

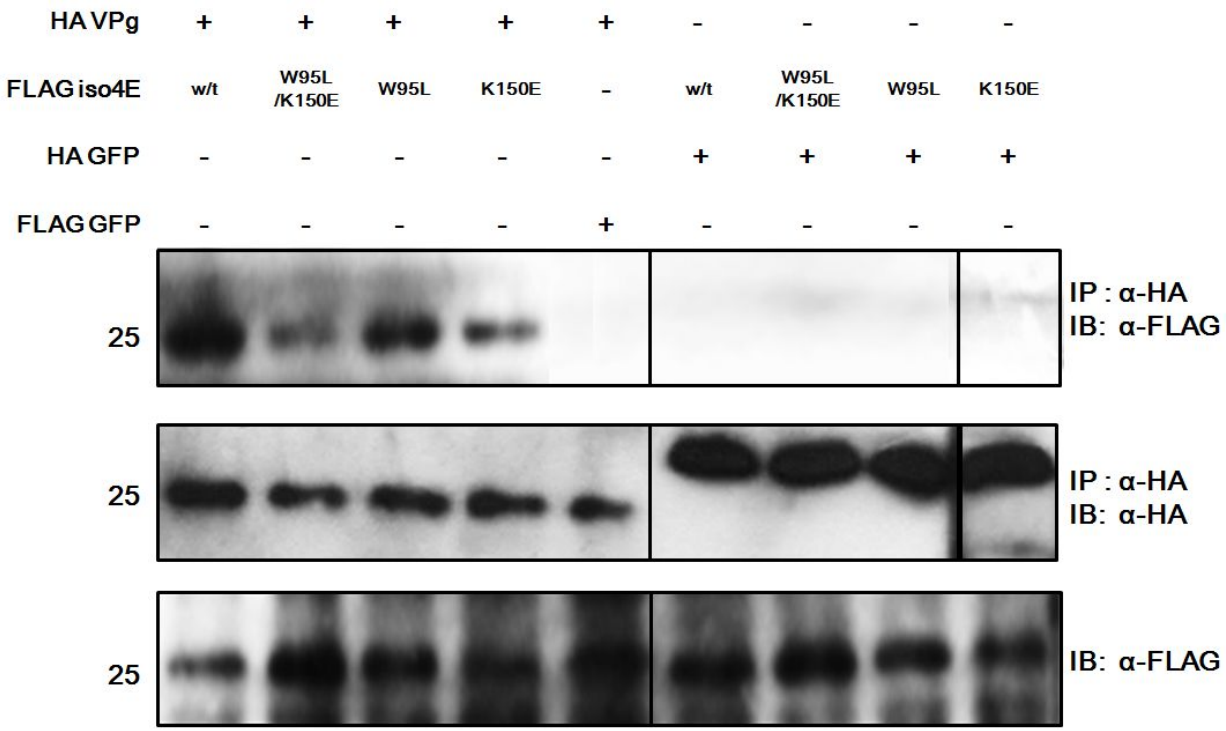

HA VPg + + + + + - - - -
FLAG iso4E w/t W95L /K150E W95L K150E - w/t W95L /K150E W95L K150E
HA GFP - - - - - + + + +
FLAG GFP - - - - + - - - -
25
IP : α-HA
IB: α-FLAG
25
IP : α-HA
IB: α-HA
25
IB: α-FLAG

Figure 3. *In vivo* interaction between TuMV VPg and Chinese cabbage eIF(iso)4E. (A) BiFC analysis of TuMV VPg and eIF(iso)4E. TuMV VPg was fused with the N-terminal fragment of YFP (TuMV VPg-YN) and co-expressed with the fusion of eIF(iso)4E with the C-terminal fragment of YFP (eIF(iso)4E YC). All confocal images were taken 2 d post infiltration and 4-week-old *Nicotiana benthamiana* plants were used. All images are projection stacks of multiple confocal sections produced with a Leica LCS-SL CLSM. Bars equal 50μm. Chl, Chloroplast autofluorescence. (B) Immunoblot image from co-immunoprecipitation assays. Co-IP assay between eIF(iso)4E (w/t and mutants) and TuMV VPg. Immunoprecipitation (IP) was carried out with anti-HA agarose beads and immunoblotting (IB) was performed with antibodies indicated above. GFP proteins (HA-GFP and FLAG-GFP) were used as negative controls.

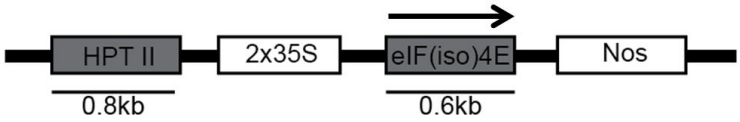
Development and analysis of transgenic Chinese cabbage over-expressing mutated *eIF(iso)4E* genes

To test the hypothesis that the eIF(iso)4E mutant that abolishes the physical interaction with VPg could induce resistance in susceptible Chinese cabbage, eIF(iso)4E proteins were over-expressed in the susceptible Chinese cabbage cultivar 'Seoul'. Genes encoding the w/t eIF(iso)4E, eIF(iso)4E W95L, W95L/K150E were transformed in the Chinese cabbage cultivar. K150L was also prepared for the transformation but it failed. Successful transgene insertion in the T_1 transformants was assessed via PCR. The hygromycin phosphotransferase (HPT) gene and the eIF(iso)4E transgenes were amplified by the PCR (Figure 4B). The amplified eIF(iso)4E transgenes were then re-sequenced (data not shown). T_1 progenies (K1-3, over-expressed w/t eIF(iso)4E T_1; K3-2, over-expressed eIF(iso)4E W95L T_1; K2-2, over-expressed eIF(iso)4E W95L/K150E T_1), which have a single copy of the transgene were selected for the further assays.

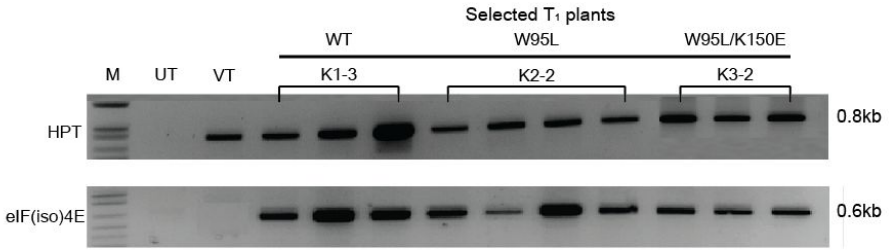
To test if the over-expressed eIF(iso)4E affects the susceptibility of Chinese cabbage, T_1 progenies were inoculated by TuMV CHN5. Untransformed plants, Vector transformed plants, eIF(iso)4E over-expressed plants which have w/t, W95L or W95L/K150E transgenes were inoculated by

TuMV. All T_1 transformants did not show developmental defects compare to the untransformed plant. The typical TuMV symptoms on the untransformed plants, vector transformed plants and over-expressed w/t eIF(iso)4E plants were observed after 20 dpi. About 35 dpi, the symptoms on the over-expressed w/t eIF(iso)4E became more severe, while the plants over-expressing W95L and W95L/K150E did not show distinct symptom (Figure 4C). The virus accumulation of these plants was assayed by DAS-ELISA (Figure 4D). This also confirmed the visual results. eIF(iso)4E w/t over-expressed plants showed that the virus particles highly accumulated in the transformants. But in W95L and W95L/K150E over-expressed plants, virus accumulation was significantly reduced compared to the w/t over-expressed transformants. According to these data, it is concluded that over-expression of mutated eIF(iso)4E can render induce TuMV resistance in susceptible Chinese cabbage.

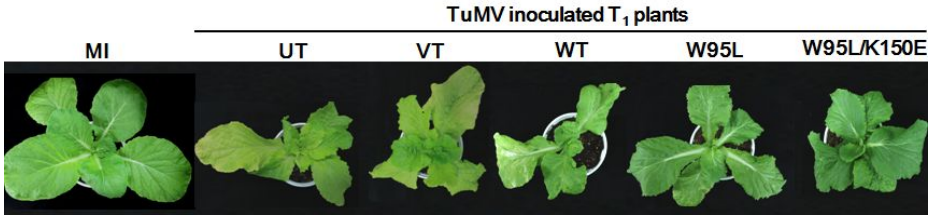
(A)


HPT II
2x35S
eIF(iso)4E
Nos
0.8kb
0.6kb

(B)


Selected T1 plants
WT
W95L
W95L/K150E
M
UT
VT
K1-3
K2-2
K3-2
HPT
0.8kb
eIF(iso)4E
0.6kb

(C)


TuMV inoculated T1 plants
MI
UT
VT
WT
W95L
W95L/K150E

(D)

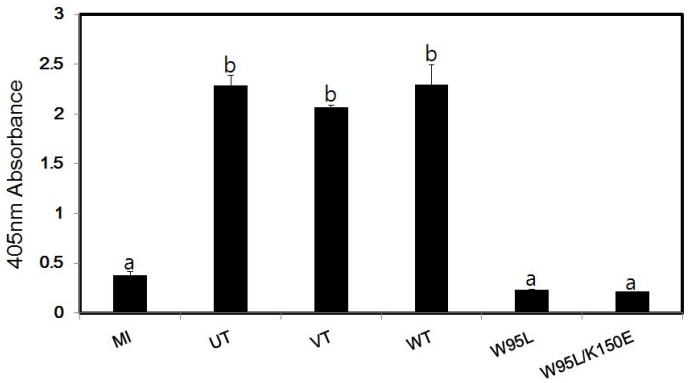
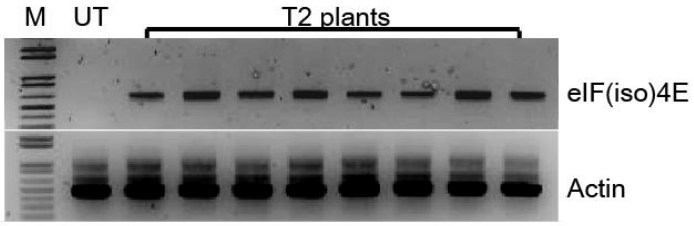


Figure 4. TuMV screening of T_1 transgenic Chinese cabbage over-expressing mutated eIF(iso)4E genes

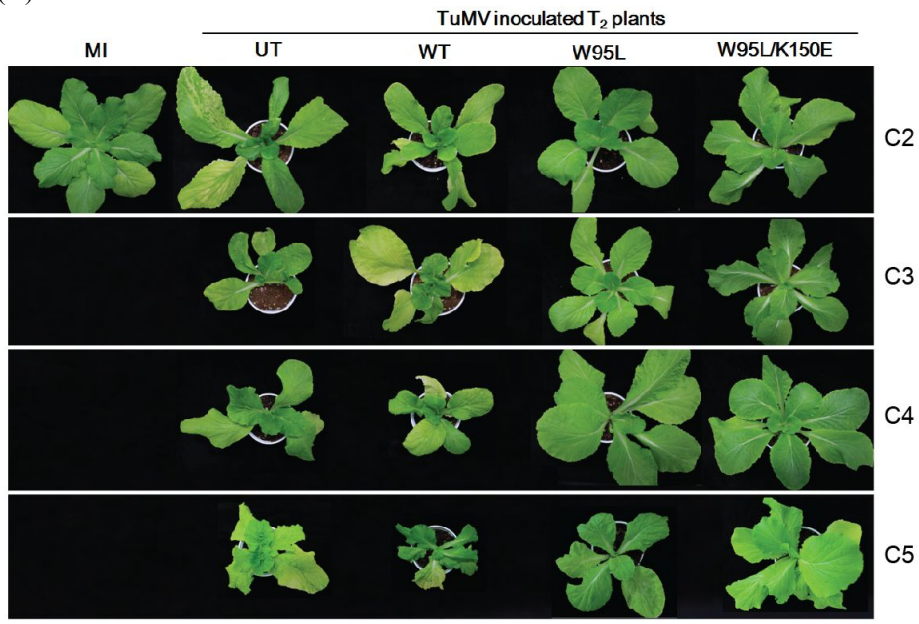
(A) Schematic diagram illustrating the pMDC32::eIF(iso)4E construct used in transformation. The expected sizes of eIF(iso)4E transgene and HPT II are shown in the picture. (B) PCR analysis to confirm the eIF(iso)4E transgene and HPT II gene using gDNA of T_1 plants. T_1 progenies that have had single copy of the transgene were selected. M, DNA marker; UT, untransformed control; VT, vector transformed control; K1-3, over-expressed w/t eIF(iso)4E T_1; K3-2, over-expressed eIF(iso)4E W95L T_1; K2-2, over-expressed eIF(iso)4E W95L/K150E T_1 (C) Phenotypes of TuMV CHN5 inoculated eIF(iso)4E T_1 plants. The plants were photographed at 35 dpi. (D) ELISA screening result of TuMV inoculation. DAS-ELISA was performed at 35 dpi to test the virus accumulation using 5-6th true leaves. MI, mock inoculated control; UT, untransformed 'Seoul' wild type control; VT, vector transformed control; WT, w/t eIF(iso)4E transformant; W95L, eIF(iso)4E W95L mutant transformant; W95L/K150E, eIF(iso)4E NR(W95L/K150L) transformant. Error bars represent standard deviation. ANOVA was performed on the data sets.

T_2 generation plants were obtained from the selected T_1 plants by self-pollination. T_2 transformants was also assessed via PCR and hygromycin selection to confirm transgenic insertion of eIF(iso)4E genes. The expression of the eIF(iso)4E transgene was also confirmed by RT-PCR (Figure 5A). The amount of endogenous eIF(iso)4E cDNA was too low to make clear band by RT PCR (30cycle). But the over-expressed plants showed uniformed bands of eIF(iso)4E transgenes. To determine if the resistance of the T_1 plants was stable in T_2 progenies, T_2 plants were inoculated with TuMV. Four strains, CHN2, 3, 4 and 5 were tested to check the resistance. The screening results of the T_2 plants were similar to those of T_1 plants. About 45 dpi, the symptoms of the T_2 plants over-expressing w/t eIF(iso)4E became clear. In contrast, the T_2 plants over-expressing W95L and W95L/K150E did not show any TuMV symptoms (Figure 5B). The virus accumulation in the transgenic over-expressed plants was also significantly lower compared to the over-expressed w/t plants. (Figure 5C, D). The TuMV screening result of T_2 transformants, showed that over-expression of the mutated eIF(iso)4E genes render resistance to all tested TuMV strains (CHN2, 3, 4 and 5). This results support hypothesis that the over-expression of mutated eIF(iso)4E is able to induce broad spectrum TuMV resistance in susceptible Chinese cabbage.

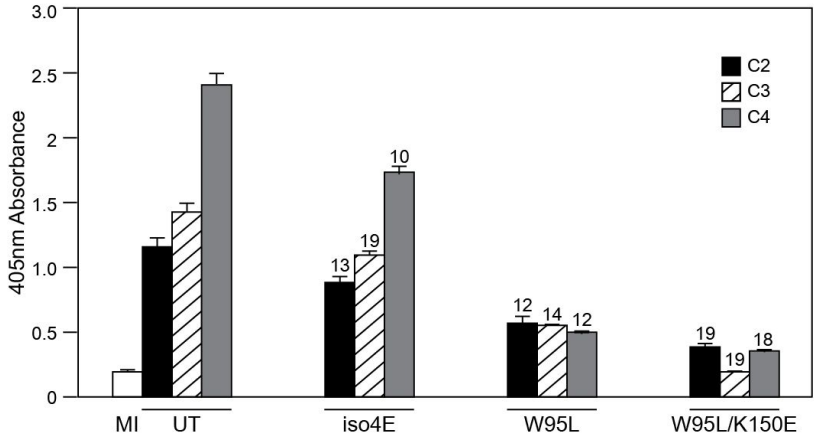
(A)


M
UT
T2 plants
eIF(iso)4E
Actin

(B)


TuMV inoculated T2 plants
MI
UT
WT
W95L
W95L/K150E
C2
C3
C4
C5

(C)



(D)

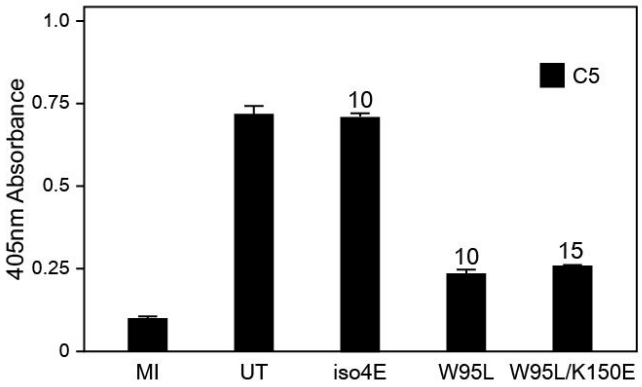


Figure 5. TuMV screening of T_2 transgenic Chinese cabbage over-expressing eIF(iso)4E mutant

(A) RT-PCR analysis to test the expression of the transgene. M, DNA marker; UT, untransformed control (B) Phenotypes of TuMV inoculated eIF(iso)4E T_2 plants. The plants were photographed at 45 dpi. (C), (D) ELISA screening result of TuMV CHN 2, 3, 4 (C) and CHN 5 (D) inoculation. DAS-ELISA was performed at 45 dpi to test the virus accumulation using 5-6th true leaves. Mock, mock inoculated; UT, untransformed 'Seoul' cultivar; WT, w/t eIF(iso)4E transformants; W95L, eIF(iso)4E W95L mutant transformants; W95L/K150E, eIF(iso)4E W95L/K150L transformants. The numbers on each bar indicate the total T_2 plants that were screened. Error bars represent standard deviation.

Yeast complementation assay

The seven candidate amino acids have their own unique physical and chemical features, and the substitution to leucine is a disfavored event in nature. This change might cause considerable changes in the cap binding ability. Hence, the yeast complementation assay was performed to test the consequences of amino acid substitutions in cap binding ability. Eight eIF(iso)4E mutants were introduced to the yeast strain JO55. The chromosomal copy of eIF4E in this yeast strain had been deleted. If the mutations in the introduced eIF(iso)4E protein impaired the interaction with 5′ cap, the yeast could not survive due to the lack of the eIF4E protein, in the absence of galactose. The eIF(iso)4E from 'Samjin' cultivar was also transformed in yeast as a positive control. After transformation, yeast cells were grown in synthetic minimal media containing 2% glucose at 30°C for four days. Yeast cells were also grown in synthetic minimal media containing 2% galactose and 1% raffinose at 30°C for four days to compare the complementation in glucose media (Figure 6). Susceptible w/t eIF(iso)4E complemented the loss of eIF4E successively. In conclusion, complementation of the eIF4E knockout yeast strain by mutated eIF(iso)4E proteins showed that all eIF(iso)4E mutants were able to complement eIF4E of yeast to some degree compared to the w/t. But the growth was not as active as for the yeast colonies in galactose media. There was

no difference in yeast growth among eight mutants and w/t. It seems that the mutated eIF(iso)4Es that may induce the TuMV recessive resistance do not lose the key function of the eukaryotic translation initiation factor, the cap-binding ability.

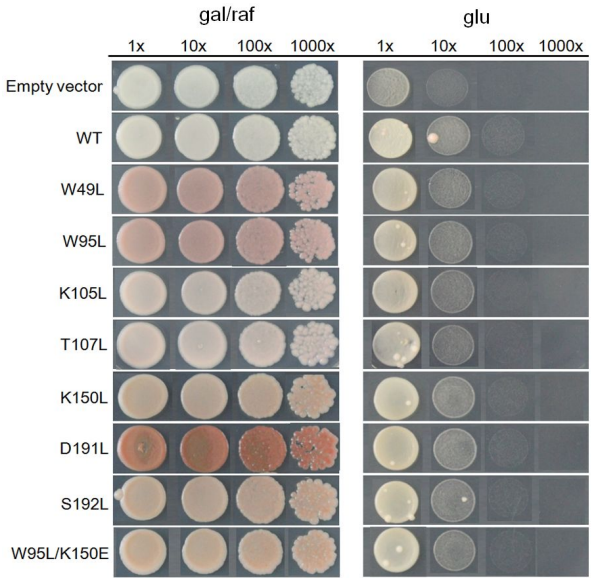


Figure 6. Complementation of yeast strain JO55 with eIF(iso)4E cDNAs.
The yeast strain JO55 was transformed with plasmids p424GBP/TRP1:eIF(iso)4E containing wild type and mutants. The yeast strain JO55 was or was not transformed with plasmids encoding wild type or mutants of eIF(iso)4E. After transformation, yeast cells were grown in synthetic minimal media containing 2% glucose. Each yeast clones were serially diluted and the growth on galactose and glucose containing media were assessed. Yeasts were incubated at 30°C for five days. WT, eIF(iso)4E w/t; W49L, eIF(iso)4E W49L mutant; W95L, eIF(iso)4E W95L mutant; K105L, eIF(iso)4E K105L mutant; T107L, eIF(iso)4E T107L mutant; K150L, eIF(iso)4E K150L mutant; D191L, eIF(iso)4E D191L mutant; S192L, eIF(iso)4E S192L mutant; W95L/K150E, eIF(iso)4E mutant (W95L/K150E). gal/raf, galactose/raffinose; glu, glucose.

DISCUSSION

In this study, transgenic Chinese cabbage resistant to TuMV was developed by overexpressing mutated eIF(iso)4E genes. Single or double amino acids of eIF(iso)4E located in the cap binding pocket were mutated. The interaction analysis via the yeast two-hybrid assay demonstrated that the single-amino acid mutants (W49, W95 and K150) and the double-amino acid mutant (W95L/K150E) of Brassica eIF(iso)4E could interrupt the interaction with the TuMV VPg. BiFC analysis and co-immunoprecipitation was also performed to ascertain the interaction *in vivo*. A yeast complementation assay showed that all eIF(iso)4E mutants retained its function. Over-expression of some eIF(iso)4E mutants induced a broad spectrum resistance against TuMV. Taken together, these data support the hypothesis that the single or double amino acid mutation in eIF(iso)4E could render TuMV resistance in *Brassica rapa*. This is the first report of engineering TuMV resistance in *B. rapa* by Brassica eIF(iso)4E over-expression.

The critical amino acids that are found in the present study are known to play an important role in formation of the cap-binding pocket of eIF4E and are highly conserved in eIF4E of many other species. Tryptophan of this amino acid position forms π-π bond with other conserved tryptophan to make an

entrance of cap-binding pocket (Rhoads et al. 2007). Lysine is known to stabilize the cap binding structure through H-bonds with phosphate group of m^7GTP (Okade et al. 2009). The function of these amino acids seems highly indispensable and mutations in this amino acid positions would be very constrained. In addition, the molecular weight or the polarity of the amino acids showed difference with exchanged amino acids. Hence, it may likely cause electrostatic and steric hindrances to the cap-binding pocket. However, in the present data, the mutated eIF(iso)4Es complemented human eIF4E that was absent in the yeast JO55. There was no differences in growth or in phenotype between transformed plants and normal plants. According to the previous research, even though the host factor loses its function, it seems that the redundancy of the host factors compensate for the mutated host factor (Duprat et al. 2002; Lellis et al. 2002; Combe et al. 2005). Most of the virus resistances that are derived by the mutation of eIF4E protein did not alter the development of the plants (Nicaise et al. 2003; Gao et al. 2004a,b; Kang et al. 2005a; Morales et al. 2005; Nieto et al. 2006; Piron et al. 2010). This supports the view that there will be nearly no phenotypic problem in applying transgenic plant in breeding.

The yeast two-hybrid data in this study supports the hypothesis that the binding site of VPg is at or near the cap-binding pocket (Leonard et al. 2000;

Miyoshi et al. 2006). But VPg seems to bind a more restricted region of eIF(iso)4E than 5′ cap as its interaction was severely affected by single amino acid mutation. According to the *in vivo* interaction analysis performed by co-IP, it seems that the mutations in W95 and K150 amino acids are effective in the restriction of VPg interaction. Though the mutations were not able to collapse the interaction, the interaction was reduced significantly especially in the mutants W95L/K150E and K150L. In the yeast two hybrid result, the β-galactosidase activity of the mutant eIF(iso)4E W95L was the lowest compare to the other mutants. But in co-IP data, the mutants W95L/K150E and K150E showed the lowest interaction with TuMV VPg. This discrepancy in the data may be due to the difference in the yeast system and the plant system.

In this study, eIF(iso)4E mutations that interrupted the interaction with potyvirus VPg induced resistance. *Braiso4Ea* copy was chosen in this study. But my previous study of TuMV recessive resistance pointed out that the *trs* recessive resistance gene is not the *Braiso4Ea* copy. But it was the only copy showing strong relationship with TuMV resistance. So the resistance gene is highly expected to be one of the other *Braiso4Ea* homologs. Based on these background reports, *Braiso4Ea* cDNA was used for this second study.

메모 [u4]: 내용 추가

The ectopic expression of mutated pepper eIF4E induced strong resistance to multiple viral species in the tomato system (Kang et al. 2007).

Another report in 2011 showed that the transgenic expression of the *pvr1*^{2} gene from pepper conferred resistance to PVY in potato (Cavatorta et al. 2011). When the *Eva1-eIF4E-1* variant which has amino acid substitutions was over-expressed in the *eIF4E-1* silenced potato, resistance against PVY was conferred (Duan et al. 2012). In my research, it looks like the over-expressed eIF(iso)4E mutant protein overwhelmed the normal eIF(iso)4E. Because of the competition between two proteins, virus-host interaction was disturbed and this procedure may lead to TuMV resistance. The transgenic protein is more abundant than the endogenous protein so that the transgenic protein overwhelms the host cell. Cavatorta et al. (2010) described about this type of resistance, which is called 'dominant negative' interference (Chandler and Werr. 2003). In this experiment, the endogenous eIF(iso)4E was not silenced in the susceptible Chinese cabbage. According to the ELISA result of TuMV screening, virus accumulation was detected in the W95L and W95L/K150E mutants, which means that there are some interactions between the endogenous eIF(iso)4E and TuMV VPg. Yeast two hybrid and co-IP data also showed that the eIF(iso)4E mutants did not abolish the interaction perfectly. This may also explain the survival of viruses in the transformants.

메모 [u5]: 내용 추가

Most of these transgenic plants engineering eIF4E showed broad-spectrum resistance (Kang et al. 2007; Piron et al. 2010; Mazier et al. 2011;

Rodríguez-Hernández et al. 2012). As the resistant spectrum is also very important in developing durable resistance, this engineering method using virus host factor is one reliable way to achieve broad-spectrum resistance. This is because most of the host factors used by plant virus are in common. It will be especially useful for crops where it is difficult to identify a resistant allele in the normal germplasm.

Here, I developed TuMV resistant Chinese cabbage by over-expression of the mutated eIF(iso)4E. This is the first report of developing transgenic TuMV resistant Chinese cabbage by eIF(iso)4E engineering. I believe that this analysis provides an important insight in the broad-spectrum resistance study of *B. rapa*. Host factors such as eIF4E and eIF4G have been known to be encoded by recessive resistance gene in many plants. This background knowledge about the two host factors assisted the significant progress in exploiting the host factors in resistant breeding. But there are not many other host factors found so far besides these factors and the pool of resistance candidate genes is too small to be utilized wildly for various crops. Although many laboratories are eager to ascertain the mechanism, it is unclear how the interaction between eIF4E and VPg is connected to the resistance. There needs to be more research concerning the interactome of potyvirus and host factors to exploit resistance traits to many other crops.

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초 록

순무 모자이크 바이러스(*Turnip mosaic virus*)는 주로 배추과(Brassicaceae)를 기주로 하여 심한 모자이크 병징과 더불어 기형, 위축, 왜화 등의 병징을 유도하여 배추 등 여러 작물에서의 피해가 보고되었다. 본 연구는 궁극적으로 배추에 많은 피해를 주는 것으로 보고된 TuMV가 어떻게 기주를 감염시키고 병징을 일으키고 식물은 어떠한 방법으로 저항성형질을 보일 수 있는지에 대한 이해를 넓히는 데에 그 목적이 있다. 제 1강에서는 순무 모자이크 바이러스에 대한 *trs* 열성 저항성의 유전 양상을 분석하고 *trs* 저항성 유전자와 배추의 해독개시 인자인 eIF(iso)4E가 강한 연관이 보이는 것을 확인하였다. 세 가지 eIF(iso)4E 인자 중 *Braiso4Ea*의 분자표지가 저항성과 공 분리하는 것을 확인하였으며 *trs*의 유전자 지도를 작성하여 그 위치를 찾았다. 제 2강에서는 eIF(iso)4E *Braiso4E*를 이용하여 TuMV에 저항성을 갖는 배추를 개발하였다. 우선 TuMV VPg 단백질과 eIF(iso)4E와의 상관관계가 저항성 기작과 연관이 있음을 확인한 후 이병성 배추에서 eIF(iso)4E 돌연변이들의 과대발현을 유도하였다. 바이러스 이병성 및 저항성 검정 결과, eIF(iso)4E의 W95와 K150 부분의 아미노산의 돌연변이가 VPg 단백질과의 상호작용을 억제하고 광범위 저항성을 유도한다는 것을 확인하였다.

주요어: *Brassica rapa*, 순무 모자이크 바이러스 (TuMV), 열성 저항성, eIF(iso)4E, 형질전환