A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Map-based Cloning of the Low Temperature Sensitive Gene *sy-2* in Pepper (*Capsicum chinense*), and the Study of Cytoplasmic Male Sterility in Transgenic Chinese Cabbage (*Brassica rapa*) Overexpressing the Pepper *Orf456* Gene

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UNDER THE DIRECTION OF DR. BYOUNG-CHEORL KANG
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Temperature is one of the main environmental factors affecting the productivity and geographical distribution of crops. Many horticultural crops originating from the tropical or subtropical regions are temperature sensitive and susceptible to low temperature injury. The pepper cultivar '*sy-2*' (*C. chinense*,

Seychelles-2) native to the Seychelles in Africa was found by Japanese researchers in 1989, exhibits developmental abnormalities when grown under temperatures lower than 24°C. Previous studies have mapped the *sy-2* gene to a 300 kb region of the Ch1_scaffold 00106 in tomato chromosome 1, but the *sy-2* gene was not formally identified. To facilitate the identification of the *sy-2* gene, fine mapping of the *sy-2* locus and map-based cloning of the sy-2 gene were performed.

In chapter I, a fine map of the *sy-2* locus was developed using two F_2 populations, and 14 SNP and four KASP markers derived from comparative analysis of genomic sequences between tomato scaffold (C01HBa0051C14) and pepper scaffold (2607 (377.77 kb), 3515 (200.85 kb), and 2510 (318.608 kb)). The *sy-2* gene was delimitated between SNP5-5 and SNP3-8 at the distal region of pepper chromosome 1 spanning the target region of 138.8 kb. Gene annotation of the region showed 27 predicted genes.

In chapter II, the predicted genes were analyzed using RNA-seq, RT-PCR and qPCR methods. RNA-seq analysis was carried out and a heat map was constructed to show differential expression of candidate genes between the wild-type and '*sy-2*' plants under cold stress conditions. The RNA-seq and RT-PCR results showed that predicted gene *ORF10* and *ORF20* were apparently down-regulated in '*sy-2*' plants. qPCR analysis was also performed to confirm *ORF10*

and *ORF20* gene expression patterns. Expression levels of *ORF10* were 8.2 times higher in wild-type plants than in '*sy-2*' plants at 20°C. DNA sequencing identified several *sy-2* specific SNPs that encode amino acid mutations C231G and N315D for *ORF10* and S256K for *ORF20*. Overall, among the predicted *sy-2* candidate genes, *ORF10* and *ORF20*, which are predicted to encode kelch type F-box proteins, were observed to be differentially expressed in wild-type and '*sy-2*' plants. Gene suppression experiments were carried out using VIGS. Following infection, plants with gene suppression of *ORF10* and *ORF20* showed moderate growth retardation of newly emerging leaves, with what appears to be similar growth characteristic to the '*sy-2*' plant. Plants grown with co-suppression of both *ORF10* and *ORF20* displayed severe growth retardation after 3 weeks, with emerging true leaves exhibiting loss of pigmentation, as well as narrow, asymmetric and thicker growth, which is similarly to the leaf growth of the '*sy-2*' plant grown at 20°C.

In chapter III, the *orf456* gene previously identified as the CMS gene in pepper was transformed to the Chinese cabbage (*Brassica rapa* L.) to gain further insights into this gene function. Among 30 T_1 transgenic Chinese cabbage lines obtained approximately 50% of T_1 transgenic lines showed male sterility. Nevertheless, how the *orf456* gene precisely functions to induce male sterility and its biochemical function remain to be discovered.

Key words: *Capsicum chinense*, Chinese cabbage (*Brassica rapa subsp. pekinensis*), Cytoplasmic male sterility, Fine mapping, Map-based cloning, Mutation, *orf456*, Temperature-sensitive.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
CAPS	Cleaved amplified polymorphic sequence
CBF	C-repeat binding factor
cDNA	Complementary deoxyribonucleic acid
cM	centiMorgan
COR	Cold regulated
COS	Conserved ortholog set
CTAB	Cetyl trimethylammonium bromide
ER	Endoplasmic reticulum
GO	Gene ontology
HRM	High resolution melting
HTP	Hygromycin B
JA	Jasmonic acid
KASP	Kompetitive allele specific polymerase chain reaction
MAS	Maker assisted selection

MS	Murashige and Skoog medium
NBSs	Nucleotide binding sites
NICEM	National Instrumentation Center for Environmental Management
ORFs	Open reading frames
PCR	Polymerase chain reaction
PMC	Pollen mother cell
PSII	Photosystem II
qPCR	Quantitative polymerase chain reaction
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Salicylic acid
SAG	Salicylic glucoside
SCF	Skp, cullin, F-box containing complex
SNP	Single nucleotide polymorphism
TRV	Tobacco rattle virus
UTR	Un-translated region
VIGS	Virus induced gene silencing

GENERAL INTRODUCTION

Low temperature stress is one of the major environmental factors that limit the geographic distribution and agricultural productivity of plants (Sanghera et al., 2011). Tropical and subtropical plants show a significant physiological dysfunction when exposed to nonfreezing temperatures of about 4-15°C, which is referred to as chilling injury, and freezing injury which is damage to plants caused by temperatures below the freezing point (Slovakova et al., 2011; Yadav, 2010). Each year, worldwide losses in crop production due to low temperature damage account to approximately US$ 2 billion (Sanghera et al., 2011).

Pepper (*Capsicum* ssp.), original from the tropics, is a very important economic crop from *Solanaceae* family grown commercially worldwide. Its optimum growth temperature lies between 25 and 30°C and changes in temperature have been shown to adversely affect the development of the pepper plants that results in a variety of developmental and physiological disorders (Pickersgill, 1971).

Physiological events related to chilling injury have been extensively described. Modifications in membrane composition, especially in the degree of saturation of their constitutive lipids, which regulate membrane fluidity and permeability, are

the first notable effects of chilling injury. In bell pepper fruits (*Capsicum annuum*), changes to cell ultrastructure was induced by chilling injury; peroxisomes and starch grains were not detected and the structure of the chloroplast was seriously damaged in chilled tissues (Sanchez-Bel et al., 2012). Exposing pepper fruits to 18°C enhanced water loss, respiration, ethylene production, and electrolyte leakage (Lim et al., 2007). A pepper species Tr-13 (*C. chinense*), which originates from Trinidad & Tobago, did not develop true leaves at 20°C (Koeda et al., 2013). An exacerbation of oxidative stress due to the overproduction of reactive oxygen species (ROS) has been considered as a secondary effect of chilling injury. ROS composed of superoxide, hydroxyls, peroxides, and oxygen singlet may contribute to the resistance to chilling injury development in pepper fruits (Finger et al., 2016). However, the molecular bases of low temperature stress, that is, perception, signal transductions and finally gene and protein expression changes induced by this environmental factor have been rarely studied in pepper.

'*Sy-2*', (*Seychelles-2*) native to the Seychelles, was found by Japanese researchers as a temperature-sensitive natural mutant of *C. chinense*. In previous studies of this mutant, in 2009, a Japanese research group suggested that '*sy-2*' plant leaves showed irregular shape and deficient development of chlorophyll when the growth temperatures were lower than 24℃ (Koeda et al., 2009). Our previous study found the chloroplast structure was impaired when the '*sy-2*' plant

was grown under 20℃ (An et al., 2011). A biochemical study of this mutant showed that ROS were highly generated in sy-2 mutant and the ROS scavenging genes might be functionally impaired. Fatty acids are deficient in the '*sy-2*' mutant (An et al., 2011). After 7 days of temperature treatment, salicylic acid (SA) and salicylic glucoside (SAG) contents were 9.5- and 3.2-fold higher, respectively, in mutant at 20℃ than 24℃ (Koeda et al., 2012). In 2012, Koeda's research group performed a transcriptome analysis of stress-inducible gene expression using microarray. Of the 411 genes that were up-regulated in response to growth at this restrictive temperature, they found that a quarter of them were defense related genes or predicted to be defense-related genes (Koeda et al., 2012). Based on the molecular mapping of the '*sy-2*' mutant, the *sy-2* gene was located at the pepper linkage group 1, 1.6 and 3.1 cM away from two COSII markers C2_At4g29120 and C2_At1g09070, respectively. The *sy-2* gene was located in a 300 kb region of the Ch1_scaffold 00106 in tomato chromosome 1 (An et al., 2011).

Based on previous research, our first study focused on a high resolution genetic map of the *sy-2* locus and the identification of candidate *sy-2* genes through a map based cloning analysis in pepper (Chapter I and II). Moreover, candidate gene's function was studied by virus induced gene silencing in pepper (Chapter II).

Hybrid vigor is a promising approach to tackle the challenge of sustaining enhanced yield gains of crops. Cytoplasmic male sterility (CMS), which is a maternally inherited trait, characterized by production of flowers with nonfunctional male gametophyte; it is interactions between mitochondrial genes and coupled nuclear genes (Schnable and Wise, 1998). CMS lines have been reported in more than 150 plant species and are used as a cost-effective system to promote efficient hybrid seed production and provide a way to probe the role of the mitochondrion in reproductive development (Bohra et al., 2016; Schnable and Wise, 1998). Many CMS genes result from rearrangements of the mitochondrial genome. Most genes belonging to the mitochondrial electron transfer chain (mtETC) pathways have been found to be associated with CMS. Among them, *cox1*, *atp8*, and *atp6* are important genes involved in the CMS (Hanson and Bentolila, 2004; Kim et al., 2006).

In a previous study, a pepper CMS candidate gene *orf456* was isolated from the pepper CMS line 'PI164835' (Jo et al., 2009; Kim et al., 2007). The Brassicaceae family has drawn the attention of the scientific community in identifying CMS genes (Budar etal., 2004). In Chapter III, this pepper CMS candidate gene *orf456* was studied in transgenic *B. napa* for gene function.

LITERATURE REVIEW

1. Pepper

Pepper (*Capsicum* ssp.) is belonged to the Solanaceae family (Pickersgill, 1971). This genus originated from Central and South America and comprises about 30 species, of which, the five main domesticated species are *C. annuum* L. (hot and sweet peppers), *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L., and *C. pubescens* Ruia. Pepper is one of the most important domesticated genera that dominate the world hot spice trade, which includes some of the hottest cultivars on earth and is grown wildly in the tropics and many temperate regions of the globe (Nicolai et al., 2013; Onus and Pickersgill, 2004). With the wild growth and production of main crops in many temperate regions of the world, crops are always challenged by abiotic and biotic stresses.

1.1 Abiotic stress

Drought, heat, cold, and salinity are among the major abiotic stresses that can adversely affect the growth and development of plants (Dwivedi et al., 2008; Ismail et al., 2007; Manneh et al., 2002; Ramegowd and Senthil-Kumar, 2015; Sheehy et al., 2005). Among these, temperature is one of the main environmental

factors affecting the productivity and geographical distribution of crops (Miura and Furumoto, 2013; Thakur et al., 2010; Theocharis et al., 2012). To mitigate or avoid potential damage caused by these stresses, plants must effectively sense, respond, and adapt to changes in their environment by altering their physiological, biochemical, and genetic characteristics (Arbona et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 2000; Wang et al., 2013).

1.2 Low temperature response in plants

Low temperature affects many plant physiological processes, such as water and nutrient uptake, photosynthesis, growth, and development (Chinnusamy et al., 2007; Harfied and Prueger, 2015). Plant species are able to alleviate the unfavorable effects of low, non-freezing temperatures through a complex adaptive mechanism known as cold acclimation, which is associated with many biochemical and physiological changes (Barah et al., 2013; Chinnusamy et al., 2003; Miura and Furumoto, 2013; Theocharis et al., 2012). Plants differ in their tolerance to freezing (<0°C) and chilling temperatures (0-15°C). While plants from temperate regions tend to be tolerant to chilling temperatures; many are sensitive to freezing temperatures. However, through a process known as cold acclimation, plants from temperate regions are able to increase their tolerance to chilling temperatures (Levitt, 1980). However, many horticultural crops

originating from the tropical or subtropical regions, such as maize, rice and tomato are temperature-sensitive, susceptible to low temperature injury (Hasanuzzaman et al., 2013) and largely lack the capacity for cold acclimation (Zhu et al., 2007). Since many of these crops are cultivated in geographical regions that do not fully satisfy the temperature preferences of the plant during the growing season, temperature represents a significant challenge in the management of such crops. As such, an understanding of the molecular mechanisms responsible for cold stress is essential to optimize crop breeding and production.

Cold acclimation by temperate plants has been shown to require marked transcriptional reprogramming to alter the expression of a wide class of genes with a wide range of biological functions (Fowler and Thomashow, 2002). A key response to cold temperatures is growth repression (Scott et al., 2004), as well as morphological, biochemical, and physiological changes such as an increase in cellular calcium contents, an accumulation of ROS and the formation of cryoprotective proteins and cryoprotective metabolites such as sugars and amino acids (Airaki et al., 2012; Cramer et al., 2011; Patel and Franklin, 2009; Ruelland et al., 2009). To understand the molecular basis of low temperature sensitivity in plants, several low temperature-sensitive mutants have been explored. The *Arabidopsis thaliana fatty acid desaturase 2* (*fad2*) mutant deficient in the endoplasmic reticulum (ER) 18:1 desaturase, shows abnormal leaves and a severe

dwarf phenotype under low temperature due to the disintegration of the cell membrane (Miquel and Browse, 1994; Zhang et al., 2012). The *Arabidopsis nonphotochemical quenching 1* (*npq1*) mutant, which is deficient in xanthophyll cycling, exhibits an accumulation of excessive oxidative stress causing the inhibition of photosystem II (PSII) at low temperature (Havaux and Kloppstech, 2001). The *tobacco temperature dependent shooty* (*tds*) mutant displays abnormal mesophyll cells, thick narrow leaves, and shorter internodes when grown at low temperature (Samuelsen et al., 1997). The *Arabidopsis bonzai1* (*bon1*) null mutants produce miniature fertile plants, and the *BON1* and *BAP1* (*BON1-associated protein)* genes were suggested to have a direct role in regulation of cell expansion and division at lower temperatures (Hua et al., 2001). The maize mutant inbred line *M11* shows remarkably lower accumulation of chlorophyll, which is associated with impaired development and function of chloroplasts (Millerd and McWilliam, 1968). The rice *low temperature albino 1* **(***lta1*) mutant seedlings has albino leaves characterized by remarkably reduced contents of chlorophyll and chlorophyll precursor molecules (Peng et al., 2012). Thus, low temperature responses in plants involve many aspects of plant physiology, many different metabolic pathways, and complex genetic interactions that make the study of a plant's response to low temperature stress challenging.

The transcriptome of plants subjected to cold stress varies markedly between plants, and large portions of the genome have been shown to be regulated by cold temperature. In *Arabidopsis*, for example, approximately 10% of genes are cold temperature regulated (Eremina et al., 2016). A significant number of these so called cold-regulated (*COR*) genes have been shown to contain the C-repeat/dehydration-responsive element in their promoters, that are bound by C-repeat binding factors (*CBF*) in response to cold and other abiotic stresses (Eremina et al., 2016). In *Arabidopsis*, the regulation of *CBF*-controlled genes has been shown to involve three, physically linked genes *CBF 1*, *2*, and *3* which act to control overlapping gene sets (Park et al., 2015). In addition, *CBF* expression is controlled by a number of transcription factors, such as the repressor *ZAT12* (Vogel et al., 2005), and the activators *ICE1* and *ICE2* (Chinnusamy et al., 2003; Fursova et al., 2009). The abundance of *ICE1*, a MYC-like basic helix-loop-helix protein gene, is controlled by phosphorylation and ubiquitination catalysed by the E3 ubiquitin ligase *HOS1* (Dong et al., 2006). In pepper (*C. annuum*), *CBFs* have also been identified and implicated in a pepper's tolerance to chilling stress (Kim et al., 2004; Yang et al., 2011). Although *CBFs* are important regulators of the cold-responsive transcriptome, other transcription factors have been shown to play a role. *ZAT10*, for example, is a negative regulator of *COR* genes whose activity is

repressed by the bifunctional enolase *LOS2*/*AtMBP-1* in response to cold stress (Lee et al., 2002).

1.3 Low temperature responses in pepper

In *C. annuum*, it has been shown that a total of 317 genes are cold temperature inducible, and notable among the up-regulated cold stress genes are a variety of transcription factors, including ethylene-responsive element binding protein (*CaEREBP-C1* to *C4*), *bZIP* (*CaBZ1*), ring domain protein *(RVA1*), heat shock transcription factor (*HSF1)* and *CaWRKY* genes (Hwang, 2005). Further studies have confirmed the role of *CaEREBP C1-C4* in cold stress tolerance, these genes are strongly induced under cold stress, and transgenic plants engineered to constitutively express these genes exhibited significantly increased tolerance to cold temperatures (Yu et al., 2013). *CaMBF1*, which encodes the coactivator multiprotein bridging factor 1, was identified in a screen for cold-related genes regulated by abscisic acid (ABA) (Guo et al., 2013 and 2014). *CaMBF1* is repressed by high salt, and when overexpressed in *Arabidopsis*, visible leaf damage and electrolyte leakage of cell damage were aggravated in response to cold stress (Guo et al., 2014). Further, overexpression of *CaMBF1* in *Arabidopsis* resulted in reduced tolerance to both cold and salt stress during seed germination and post-germination stages (Guo et al., 2014). Moreover, several genes that are

induced in response to temperature stress are also known to be induced by salt stress, such as *CaEREBP-C3*, *CaBZ1*, a putative auxin-repressed protein, *CaTPP1*, *LEA5* (Hwang, 2005), and *CaBl-1* (Isbat et al., 2009). Taken together, these studies suggest a certain amount of crosstalk between abiotic stress response pathways.

1.4 Mechanism of low temperature response in plants

It is believed that cold is first sensed through receptors on the membrane of plant cells (Yadav, 2010). Cold-sensitive calcium channels have been identified in plants and postulated to be the primary sensor of cold, but no such channel has yet been identified at a molecular level (Knight and Knight, 2012). At a cellular level, low temperatures cause a reduction in membrane fluidity and this membrane rigidification effect is thought to be sensed and activate *COR* genes (Knight and Knight, 2012; Yadav, 2010). Although this membrane fluidity has been shown to be a sensing mechanism in plants, there is evidence that temperature can also be sensed through chromatin remodeling (Kumar and Wigge, 2010; Kumar et al., 2012) and pre-mRNA splicing (Capovilla et al., 2015).

Regardless of the mechanism by which cold is perceived, the cold temperature signal must be transduced to the nucleus. It has been shown that this is accomplished through a complex interaction of signal transduction pathways that

involve calcium, ROS, lipid signaling cascades (Yadav, 2010), and protein phosphorylation cascades (Barrero-Gil and Salinas, 2013; Knight and Knight, 2012). Through such signaling pathways, plant cells are able to regulate transcription factors of cold response genes.

Recently, a large body of evidence also supports the role of phytohormones as key components in these pathways which serve to link and regulate stress adaptive signaling (Kazan, 2015). A link between phytohormones, such as ethylene (Cheng et al., 2013; Yu et al., 2013), ABA, jasmonates (Dar et al., 2015), SA (Khan et al., 2015), and gibberellins (Colebrook et al., 2014) and abiotic stress signaling in plants is becoming increasingly apparent. In particular, jasmonates have been shown to act in unison with signal transduction pathways involved in abiotic stress response and regulate the C-repeat binding factor pathway during cold stress (Kazan, 2015). Moreover, jasmonic acid has been shown to counteract chilling stress by activation of ROS avoidance enzymes, and also plays a role in the regulation of thermotolerance (Sharma and Laxmi, 2016).

Key mediators in response to abiotic stress in plants have been shown to be a family of F-box proteins that are substrate recognition components of the Skp1-Rbx-1-Cul1-FBox protein (SCF) ubiquitin ligases (Lee et al., 2011; Lyzenga et al., 2012). Through the modulation of amounts of regulatory proteins, ubiquitination plays a central role in the transcriptional regulation of genes required for adaption

to abiotic stresses (Lyzenga et al., 2012). In plants, the F-box proteins belong to one of the largest multigene families and control important processes in plant growth and development. Plant hormone sensing appears to be tightly regulated by such complexes and several F-box proteins have been identified that are integral to the sensing of auxin (Dharmasiri et al., 2005; Kepinski and Letser, 2005; Ruegger et al., 1998), jasmonate (Xie et al., 1998), ethylene (Kepinski and Letser, 2005; Potuschak et al., 2003) and gibberellin (McGinnis et al., 2003; Sasaki et al., 2003). Moreover, F-box proteins appear to be involved in plant defense responses (Kim et al., 2002), flower development (Ni et al., 2004; Samach et al., 1999), leaf senescence (Woo et al., 2001), and lateral root development (Coates et al., 2006).

Central to the regulation of these diverse processes is the ability of the F-box proteins to form a complex with the other major components of the SCF and direct ubiquitination of the substrate for degradation by the 26S proteasome (Kato et al., 2010; Smalle and Vierstra, 2004; Vierstra, 2003). Genome-wide analysis of plants has shown that they contain a large number of F-box proteins consistently with the role they appear to play in a wide variety of plant developmental processes. *Arabidopsis* has been shown to contain at least 694 different F-Box proteins (Vierstra, 2003), and rice at least 687 potential F-box proteins (Jain et al., 2007). Coupled with the diverse array of C-terminal domains within this F-box

family, such as Kelch domains, DEAD box, leucine-rich repeats, Armadillo, as well as distinct Cullin and Skp subunits potentially allows plants to generate a large number of SCF subtypes with unique substrate specificity (Kim et al., 2013; Vierstra, 2003). Typically, substrate recognition by many Cullin-based E3 ligases requires substrate modification through phosphorylation, which further suggests the role of kinases in the modulation of targets by SCF complexes.

The *C. chinense* cultivar '*sy-2*' is a local pepper landrace from the Seychelles that was previously found to be temperature-sensitive, showing developmental abnormalities when grown at temperatures lower than 24°C (Koeda et al., 2009). Transcriptome analysis of the '*sy-2*' cultivar in response to temperatures below 24°C showed that a quarter of the up-regulated genes were defense related or predicted to be defense related (Koeda et al., 2012; 2013). However, the precise molecular and biochemical basis of the cold temperature sensitivity in '*sy-2*' plants is not clearly understood. We have previously shown that a single recessive gene localized to the 300 kb region of the tomato Ch1_scaffold 00106 controls the '*sy-2*' phenotype. However, the exact location of the corresponding genomic region in pepper was not determined (An et al., 2011). This study aims to identify the *sy-2* candidate genes, with the ultimate goal to elucidate the molecular mechanism responsible for the temperature sensitivity of the '*sy-2*' cultivar.

2. Cytoplasmic male sterility

Cytoplasmic male sterility (CMS) is defined as maternally inherited inability to produce functional pollen (Bergman et al., 2000). The CMS-associated genes were cloned in diverse plant species including sugar beet, maize, wheat, rice, rapeseed, and radish (Chowdhury and Ahmad, 2010; Hanson and Bentolila, 2004; Schnabel and Wise, 1998). Most of them were chimeric genes that were composed by the fusions of sequences from other part of mitochondrial genome or sequences with unknown origin. In a number of cases, transcripts originating from these altered open reading frames are translated into unique proteins that appear to interfere with mitochondrial function and pollen development (Yamamoto, 2008). Many of CMS-associated genes are known to associated with ATP synthase relative subunits, for example, *Brassica orf222* and *pcf* gene in petunia (Hanson and Bentolila, 2004; Yang and Zhang, 2007; Yang et al., 2010). In this study, we transformed a pepper CMS gene *orf456* to a Chinese cabbage line to study the function of this *orf 456* gene (Kim et al., 2007).

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CHAPTER I

Fine Mapping of the Low Temperature Sensitive Gene *sy-2* in Pepper (*Capsicum chinense*)

ABSTRACT

Seychelles-2 (‘*sy-2*’) is a temperature-sensitive natural mutant of *Capsicum chinense,* which exhibits an abnormal leaf phenotype when grown at temperatures below 24°C. The ‘*sy-2*’ phenotype has been shown to be controlled by a single recessive gene, *sy-2*, located on pepper chromosome 1. In the present study, a high-resolution genetic and physical map for the *sy-2* locus was constructed using two individual F_2 mapping populations derived from two *C. chinense* ‘No.3341’ (from Bolivia) and the mutant ‘*sy-2*’ (from Seychelles). Using sequences between two COSII markers C2_At4g29120 and C2_At1g09070 obtained from three non-overlapping pepper scaffolds 2607, 3515, and 2510, and a pepper bacterial artificial chromosome library, fourteen single nucleotide polymorphism (SNP) markers and four kompetitive allele specific polymerase chain reaction (KASP) markers were newly developed in a 4.7 cM interval. The genetic order of all markers was completely consistent with their physical order. By comparing high-resolution melting analysis and KASP assays with the *sy2* phenotype data, the *sy-2* gene was shown to be located between SNP markers SNP 5-5 and SNP 3-8 in an interval of 0.14 cM at the distal portion of chromosome 1. Gene annotation analysis of the 138.8 kb region predicted 27 genes.

INTRODUCTION

Pepper (*Capsicum* ssp.) belongs to the Solanaceae family (Onus and Pickersgill, 2004). Among species of chili pepper, *Capsicum chinense* Jacq is commonly known as "yellow lantern chili" which has been domesticated in the lowlands east of the Andes (Andrews, 1995; Pickersgill, 1969). It is one of five domesticated *Capsicum* species that are grown wildly in the tropics and many temperate regions of the globe (Nicolai et al., 2013; Onus and Pickersgill, 2004). Crop production is continually challenged by abiotic and biotic stresses. Plants must effectively sense, respond, and adapt to changes in their environment (Arbona et al., 2013) by altering their physiology to mitigate any adverse effects caused by these stresses (Shinozaki et al., 2000; Thomashow, 1999; Wang et al., 2013). Among abiotic stresses, temperature is one of the main environmental factors affecting the productivity and geographical distribution of crops (Thakur et al., 2010; Theocharis et al., 2012; Thomashow, 1999).

Peppers thrive on full sun and warm weather, well-drained soil and modest fertility. Peppers have an optimum growth temperature of between 25 and 30°C, and changes in temperature have been shown to adversely affect the development of the pepper. In 1985, Japanese researchers collected *Capsicum* accessions from the Seychelles area and Caribbean countries, such as Trinidad & Tobago,

Barbados, Jamaica, Antigua & Barbuda, and Guyana (Koeda et al., 2009, 2013). The Seychelles (4-10 °S and 55-56 °E) have a mild climate in the Western Indian Ocean, with average temperatures ranging from 24 to 31°C during the year (Swabey, 1970). When a pepper cultivar *C. chinense* cv. Seychelles-2, '*sy-2*' native to the Seychelles was grown at Kyoto (35 °N, 135 °E) in Japan, they found that the newly developing leaves of all '*sy-2*' seedlings grown in spring and autumn showed abnormal morphology, but those plants leaves in summer were normal (An et al., 2011; Koeda et al., 2009, 2010). Based on these observations, the *C. chinense* cultivar '*sy-2*' was found as a natural mutant, which was previously reported to be a temperature sensitive accession (An et al., 2011; Koeda et al., 2009, 2012).

To understand the mechanism of how this '*sy-2*' mutant plant responds to the low temperature, morphological, anatomical, biochemical, and genetic studies were performed (An et al., 2011; Koeda et al., 2009). Under low temperature conditions, the '*sy-2*' cultivar exhibits abnormal leaf growth, characterized by the development of thicker and narrower cotyledons that have fewer palisade cells in the leaf-length direction, and more cells in the leaf-thickness direction (Koeda et al., 2009). The '*sy-2*' plant also shows a chlorophyll deficiency due in part to abnormal chloroplast structures and cell collapse. An excessive accumulation of ROS causing cell death has been detected in the chlorophyll-deficient sectors of

the leaves in '*sy-2*' plants grown at 20°C. The analysis of the fatty acid content of these growth impaired leaves has shown an impaired pathway of linoleic acid (18:2) to linolenic acid (18:3) (An et al., 2011). To reveal the differential gene expression of the '*sy-2*' cultivar at temperature conditions below 24°C, a transcriptome analysis of stress-inducible gene expression using microarray analysis has been performed (Koeda et al., 2013). Of the 411 genes that were upregulated at this restrictive temperature, they found that a quarter of them were defense related genes or predicted to be defense-related genes. Moreover, accumulation of high levels of SA in '*sy-2*' grown at 20°C suggested that the defense response was activated in the absence of pathogens (Koeda et al., 2013).

Genetic maps provide a powerful tool for gene identification, characterization, and utilization. Fine-mapping involves the identification of markers that are tightly linked to a targeted gene. With a fine map, marker-assisted selection (MAS) can be done (Mohan et al., 1997). The MAS is considered to be an economically efficient approach which can reduce time-consuming and expensive selection cycles. Finally, fine mapping a gene is an essential step in map-based gene isolation.

We have previously shown that a single recessive gene localized to the 300 kb region of the tomato Ch1_scaffold 00106 controls the *sy-2* phenotype. However, the exact location of the corresponding genomic region in pepper was not

determined (An et al., 2011). Based on previous work, a fine mapping in the present study was constructed by using two F_2 populations, derived from crossing '*sy-2*' and the wild type *C. chinense* 'No.3341'.

MATERIALS AND METHODS

Plant materials and growth conditions

Two accessions of pepper, *C. chinense* '*sy-2*' and *C. chinense* 'No.3341' were used as parental lines (Koeda et al., 2009). The *C. chinense* accession '*sy-2*' is a natural mutant line known to contain a single recessive gene that responds to temperature change (An et al., 2011). Two mapping populations, consisting of 1,020 (in 2012) and 1,433 individuals (in 2014), were constructed from a cross between *C. chinense* '*sy-2*' and *C. chinense* 'No.3341' and used for linkage analysis. These materials were used to confirm the linkage of SNP markers with the *sy-2* phenotype.

For the genetic analysis, all seeds were sterilized and germinated in an incubator at 30°C in darkness. One week old seedlings were transferred to 28°C chambers with 16 h light and 8 h dark cycles until the cotyledons were fully expanded. The seedlings were then transferred to 20°C chambers, and a low-temperature hypersensitivity phenotype was screened after 14 days.

Genomic DNA extraction

Two to three young leaves from plants of each accession were used for DNA extraction. Total DNA was extracted with a cetyl trimethylammonium bromide

(CTAB) procedure (Hwang et al., 2009). The concentration and purity of DNA samples was measured using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). Genomic DNA samples from two mapping populations were used to map gene *sy-2* using SNP and KASP markers.

Comparative map and sequence analysis

Based on the map position of the *sy-2* locus on chromosome 1 (An et al., 2011; Koeda et al., 2012, 2013), the COSII marker C2_At1g09070 and COSII marker C2_At4g29120 were estimated to be about 3.1 cM and 1.6 cM, respectively, from the *sy-2* gene. Those two COSII markers are located at 3.7 cM and 4.0 cM on chromosome 1 of Tomato-EXPEN 2000 (http//:solgenomics.net).

A tomato scaffold sequence (C01HBa0051C14) of 126,295 bp in length, covering the corresponding region of the *sy-2* locus, was identified based on the sequence of the C2_At1g09070 and C2_At4g29120 markers. The gene coding regions of the tomato scaffold were predicted by FGENESH (http://linux1.softberry.com). The predicted amino acid sequences were used to search for the annotated genes using the BLASTP program (http://www.ncbi.nlm.nih.gov). The sequences of gene coding regions were used to search for the homologous pepper sequences from *C. annuum* BAC database (http://cab.pepper.snu.ac.kr).

Three pepper scaffolds sequences, scaffold 2607 (377.7 kb in size), scaffold 3515 (200.8 kb in size), and scaffold 2510 (318.6 kb in size), were identified in the *C. annuum* CM334. V1.5 scaffolds database (updated on January 06, 2014).

Development of SNP markers

PCR primers were manually designed in intergenic regions of pepper sequences, based on the three pepper scaffolds, using Primer Select program (DNAStar, Inc., Madison, WI, USA) with PCR products of about 1 to 1.2 kb. PCR was carried out on a thermocycler (My Cycler™, BioRad, Hercules, CA, USA). PCR was performed in a total volume of 50 μL containing 10 μL of DNA (10 ng/μL), 5 μL of 10× Ex Taq PCR buffer (TaKaRa, Tokyo, Japan), 4 μL of 10 mM dNTP mixture (TaKaRa, Tokyo, Japan), 0.4 μL of Ex Taq polymerase (TaKaRa, Tokyo, Japan), 2 μL of 10 pmol/μL each primer and 26.6 μL of distilled water. PCR conditions involved denaturing the DNA for 4 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55-58°C, and 40 s at 72°C, then a 10 min extension time at 72°C. The PCR products were separated on a 1% agarose gel in 0.5× TAE containing ethidium bromide and visualized under UV light. The amplified bands were excised and purified with a Zymoclean PCR Purification Kit following the manufacturer's protocol (Invitrogen Korea, Seoul, Korea). Purified PCR products were sequenced at the National Instrumentation Center for

Environmental Management (NICEM) Seoul National University, Seoul, Korea. Nucleotide sequences were aligned using the SeqMan program (DNAStar, Inc, Madison, Wisconsin, USA) to detect SNP(s) position.

BAC library screening and sequence analysis

To explore and develop more closely linked markers for gene *sy-2*, a BAC library with 12× genome coverage (99%) which containing 235,000 clones with an average insert size of 130 kb constructed from *Capsicum annuum*,'CM334', was used for this study (Yoo et al., 2003). Eight BAC library screening PCR primers were designed based on end-sequences of the three pepper scaffolds. Primer sequences are listed in Table 1-2. A total of twenty-one positive BAC clones were selected for BAC end sequences at NICEM by using SP6 and T7 markers. The BAC end sequences locating at extended regions were used to search for homologous pepper sequences from the *C. annuum* BAC database (http://cab.pepper.snu.ac.kr). Finally, four BAC clones were fully sequenced by NICEM. The BAC contig was assembled (Macrogen, Seoul, Korea) using a fingerprinting contig program. Repeat sequences were filtered by RepeatMasker (http://www.repeatmasker.org) and JDotter (http://athena.bioc.uvic.ca). The gene coding regions were predicted with FGENESH (http://linux1.softberry.com) or

GENESCAN (http://genes.mit.edu) and BLASTX (E value < 3× 10-15) to distinguish exon and intron regions of fully sequenced BAC clones.

Genotype analysis by HRM and KASP assays

Genotype screening was performed using a HRM analysis method. Primer sequences are listed in Table 1-2. HRM was performed on a Rotor-Gene™ 6000 thermocycler (QIAGEN, Hilden, Germany) in 20 μL reaction mixtures containing 10× PCR reaction buffer (100 mM KCl, 20 mM Tris-HCl (Ph 8.0), and 2.5 mM $MgCl_2$), 0.25 mM of each dNTP, 5 pmol of each primer, 1 unit *Taq* polymerase, 1.25 μM Syto9 (Invitrogen, Waltham, Massachusetts, USA), and 50 ng gDNA. Cycling conditions were at 95°C for 4 min, followed by 95°C for 20 s, 58°C for 20 s, and then 45 cycles of 72°C for 40 s. HRM was run for every increment of 0.1°C between 70°C and 90°C. The screened polymorphic markers were selected for sequencing to identify the SNP(s) positions detected by HRM analysis. Linkage analysis of the markers developed in this study using CarthaGene software (de Givry et al., 2005). KASP assays were developed and four KASP markers were developed used to screen 1433 F_2 individual lines. Primer sequences are listed in Table 1-2. KASP assays were run in a 10 μL reaction system, including a 5 μL KASP Master Mix (KBiosciences, Herts, England), 0.14 μL of primer mix and 5 μL of 5-50 ng/μL genomic DNA. The PCR conditions for

KASP marker assay was 94°C for 15 min, followed by 10 cycles of touch down PCR from 61°C to 55°C with 0.6°C decrease per cycle, then followed by 26 cycles of 94°C for 20 s and 55°C for 1 min. Conditions for further thermal cycling of the KASP chemistry was 3 cycles of 94°C for 20 s and 57°C for 1 min. PCR fluorescent endpoint readings were performed using the Light Cycler® 480 Real-Time PCR System (Roche, Basel, Switzerland).

RESULTS

Inheritance analysis of low temperature sensitivity

The '*sy-2*' cultivar exhibits abnormal leaf growth at 20°C, characterized by the development of irregularly shaped, thicker and smaller leaf area compared to true leaves that developed at 28°C (Koeda et al., 2009, 2012). The F_1 and F_2 individuals, which were derived from a cross between *C. chinense* '*sy-2*' (from Seychelles) and *C. chinense* 'No.3341' (from Bolivia) were evaluated for response to low temperature after 2 weeks in cold chamber at 20°C (Fig. 1-3a). All ten F_1 plants showed a normal phenotype. The 1,020 F_2 plants segregated 788 normal and 232 abnormal phenotypes, which fits a 3:1 ratio ($\chi^2 = 2.77$, $p = 0.10$), confirming the recessive nature of *sy-2* (Fig. 1-1, Table 1-1). These results were consistent with earlier studies (An et al., 2011; Koeda et al., 2013).

Table 1-1. Phenotype segregation analysis of *C. chinense* 'No.3341', *C. chinense* '*sy-2*', and their F_1 and F_2 populations.

Population	No. of plants			Expected ratio (W:M)	χ^2	*p*
	Total	Wild-type (W)	Mutant (M)			
'No.3341'	10	10	0	-	-	-
'*sy-2*'	10	0	10	-	-	-
F_1 ('No.3341'×'*sy-2*')	10	10	0	1:0	-	-
F_2 ('No.3341'×'*sy-2*')	1,020	788	232	3:1	2.77	0.10

Synteny analysis between the pepper genetic map and the tomato

The *sy-2* gene has been previously mapped to the long arm of tomato and pepper chromosome 1 between the markers C2_At1g09070 and C2_At4g29120 (An et al., 2011). A complete map of the pepper genome was constructed based on orthologous markers that were already mapped in the tomato genome (Wu et al., 2009). This high resolution COSII synteny map provides a platform for cross-reference of genetic and genomic information between pepper and tomato. In addition, the hot pepper genome (Mexican landrace of *C. annuum* cv. CM334) has been sequenced by an international group of scientists from Korea, Israel, and USA (Kim et al., 2014). In Fig. 1-1, a tomato scaffold sequence (C01HBa0051C14) of 126,295 bp in length covering the corresponding region of the *sy-2* locus was identified based on the sequences of C2_At1g09070 and C2_At4g29120 markers obtained from Sol Genomic Network (SGN) (http//:solgenomics.net). This tomato sequence was used to BLAST with a *C. annuum* CM334. V1.5 scaffolds database (updated on January 06, 2014) to search for homologous pepper sequences (http://cab.pepper.snu.ac.kr). There were three non-overlapping pepper scaffold sequences, scaffold 2607 (377.772 kb in size), scaffold 3515 (200.85 kb in size), and scaffold 2510 (318.608 kb in size) identified in the *C. annuum* CM334. V1.5 scaffolds database (http://cab.pepper.snu.ac.kr).

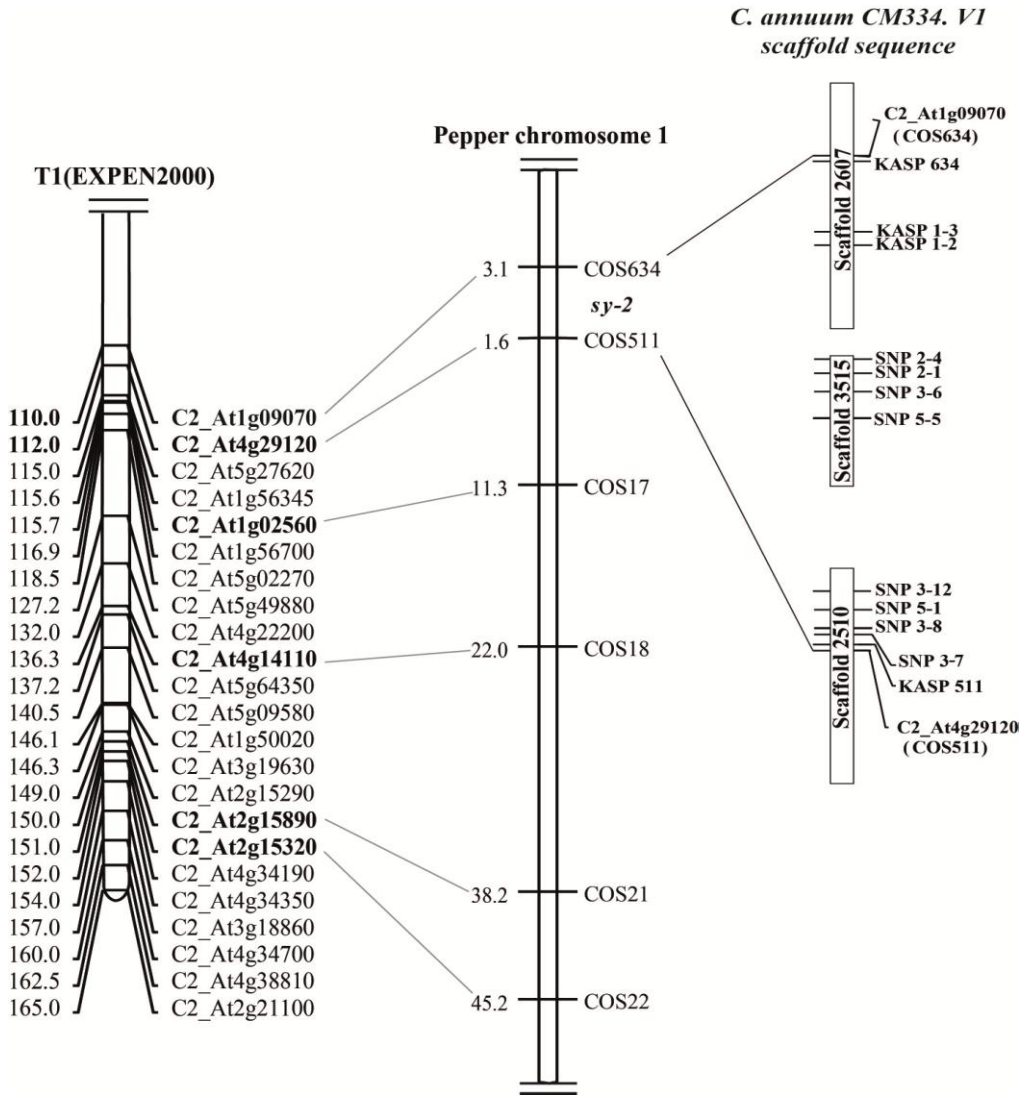


Fig. 1-1. Linkage map of the *sy-2* locus in *C. annuum* and tomato chromosome 1 distal region. The location of the *sy-2* locus on pepper chromosome 1 between COSII marker C2_At1g09070 and C2_At4g29120 is based on An et al., (2011). Markers on the tomato EXPEN2000 chromosome 1 and pepper chromosome 1 are partially shown here. Eight SNP markers and four KASP markers that were linked to the *sy-2* locus are indicated next to the *C. annuum* scaffold region. Numbers on the left indicate genetic distances (cM).

Marker development

In our previous study, to determine the chromosomal location of the *sy-2* gene, 91 COSII markers were used to map 12 pepper linkage groups in an F_2 population (total 108 individuals) derived from a cross between wild-type 'No.3341' and mutant '*sy-2*'. Among them, six COSII markers (COS634, COS511, COS17, COS18, COS211, and COS22) were found to be linked to the *sy-2* gene (An et al., 2011). The closest flanking markers, COS511 and COS634, were approximately 1.6 and 3.1 cM away from the *sy-2* locus, respectively (Fig. 1-1). Since relatively a few markers have been mapped on tomato and pepper chromosome 1 between these two markers, a high-resolution genetic map was generated for the *sy-2* locus using eight SNP markers (HRM based) and four KASP markers. SNP markers were designed in intergenic regions based on sequence information derived from the three CM334 scaffold sequences, scaffold 2607, scaffold 3515, and scaffold 2510. Details of these markers are shown in Table 1-2. Our initial mapping effort identified 16 new markers for the *sy-2* locus from which twelve markers were used for genotyping: three KASP markers (KASP 634, KASP 1-3, and KASP 1-2) from scaffold 2607, four SNP markers (SNP 2-4, SNP2-1, SNP 3-6, and SNP 5-5) from scaffold 3515, and one KASP marker (KASP 511), and four SNP markers (SNP 3-12, SNP 5-1, SNP 3-8, and SNP 3-7) from scaffold 2510.

Table 1-2. SNP markers used in fine mapping of temperature-sensitive gene *sy-2* in *C. chinense*.

KASP markers	Name	Allele specific primer	Common primer	SNP	Amplicon (bp)
	KASP 1-2	GAGGAGTTGTTTTAATCTTGAGTAGGA/G	GTCCATATCATTACTTTAGAAGTCAGACAA	A/G	-
	KASP 1-3	AATCTTCCTCCCAGGATAAAATGGC/T	AAGAGGCACATGTACCTTTTAAGTGAGAA	C/T	-
	KASP 634	GTACGGGTGAAGATAGTGAGAGC/T	CGTTCTATGTGCGCCCACTAATCAA	C/T	-
	KASP 511	CAAGAGCTTCTTTTTGAATAGTCACC/A	CGAAGCACTTGTAGAAGTTAGTAGACATA	C/A	-
HRM markers	Name	Forward primer	Reverse primer		Amplicon (bp)
	SNP 2-1	GAAAACCTATAAAGTTGCTAACCC	CATGTTAGTCAATTTGCTCCCC	A/C	157
	SNP 2-3	CAATACAGTCTACCAAACGATCCC	CCCTTTTACCCGCCTTTGCC	A/G	244
	SNP 2-4	GAAGTCAAGTGTTTGCGGTG	CTTTTGATGTTTGTCTGGCTGC	T/C	162
	SNP 3-1	CTGTATCTGTCCTGAGGCGG	GCAATGGGTTGTCTGGGAGG	A/G	231
	SNP 3-2	CATTCATTCATTCATCAGTCCAGC	CTAGCTTTACTTATTTCCCCCAG	G/A	352
	SNP 3-6	GTTTTTGGAGGATCTGACACG	CTGCACCCTTGAACTTATCTCG	A/C	260
	SNP 5-1	GGTGGACAATCTCAGTCAGTCG	CACTTAGGAATCGTTTGGTAGGC	T/C	171
	SNP 6-16	CTCATCATTTCAACAAGTTTGGGG	TGAACACTACAAAGTGAACGAATCG	C/T	127

SNP markers	Name	Forward primer	Reverse primer		Amplicon (bp)
	SNP 3-7	TGGGATGAAGATGATGATGAGTGG	ACCTTATGATACATCAGCAGAAGCAG	A/G	1219
	SNP 3-8	TGTAACATCCCTCTGGTACTCG	AAGAGAGAGATGAAAAGAGAAAGAGG	A/G	1563
	SNP 3-12	AGAACAAGATGAATATTTAAAGGTTGGC	GAGAGAGTAATTCTTATTTCTTCTTTTGAGG	A/G	1403
	SNP 3-13	AACTTACTTTCTATACTTTATCACATTTTACCG	AGCAAGCTGAGTTCGTTGTCG	A/G	923
	SNP 5-3	TGGATATTTGGACGATTACAAAAGCAC	ACTTGGGATCCTTCTAGATAAAGTGC	C/G	1102
	SNP 5-5	AGCATTTGGAAGAATTATGATTGGAG	AGGCAAAATTTGTTGAAACGGTC	A/G	1294

BAC library screening and marker enrichment

BAC end sequences were also explored for developing more closely linked markers for the *sy-2* locus. In this study, seven BAC end markers (gap 2-1, gap 3-1, gap 3-3-2, gap 3-6, gap 4-1, gap 4-3, and gap 4-5) were developed for screening the BAC library (Table 1-3). This BAC library with 12× genome coverage (99%) was used for initial screening from *C. annuum*, 'CM334' (Yoo et al., 2003).

This screening yielded fourteen positive BAC clones (422K18, 547H22, 534N9, 343L4, 331K22, 551K8, 551I9, 564P1, 679P23, 286C6, 555C12, 319A14, 586F23, and 444J2) in Fig 1-2. Of four BAC clones (422K18, 547H22, 534N9, and 343L4), which overlapped to cover the gap between two non-overlapping scaffolds, scaffold 3515 and scaffold 2510, containing the *sy-2* genes. Those four BAC clones were completely sequenced by NICEM. Based on BAC clones sequencing results, two additional new SNP markers (SNP5-3 and SNP 3-13) were developed for genetic mapping.

Table 1-3. Primer sequences used for BAC library screening.

BAC-end primer	Primer sequence	Amplicon (bp)
gap 2-1	F: CACATAAATTGGACAAAGGGAG	267
	R: GGAATCACGATACTATTTACCC	
gap 3-1	F: GTTCACCTTGATAGAGCAC	299
	R: GATCAACGCTCGTATTTCCC	
gap 3-3-2	F: CACTTAGACCCCAACCCAGC	1556
	R: CCTCGGATCATTAAGATTTGCGGAC	
gap 3-6	F: GGGTTCACATGACAAGTGCG	303
	R: GTAGTAGACGATGTCTTCCAG	
gap 4-1	F: GCTGCAGTTGAGTATGTGGTC	363
	R: CTCTTGATGATTCATCTTTCGC	
gap 4-3	F: CTTACACTTCAGAATCCCAG	1459
	R: CATGGATCTAACTTGACGGAGG	
gap 4-5	F: CGCGAAGTCTTCAAATAATGCC	1139
	R: GACACTCAAAGCAAACATCCG	

F, forward; R, reverse.

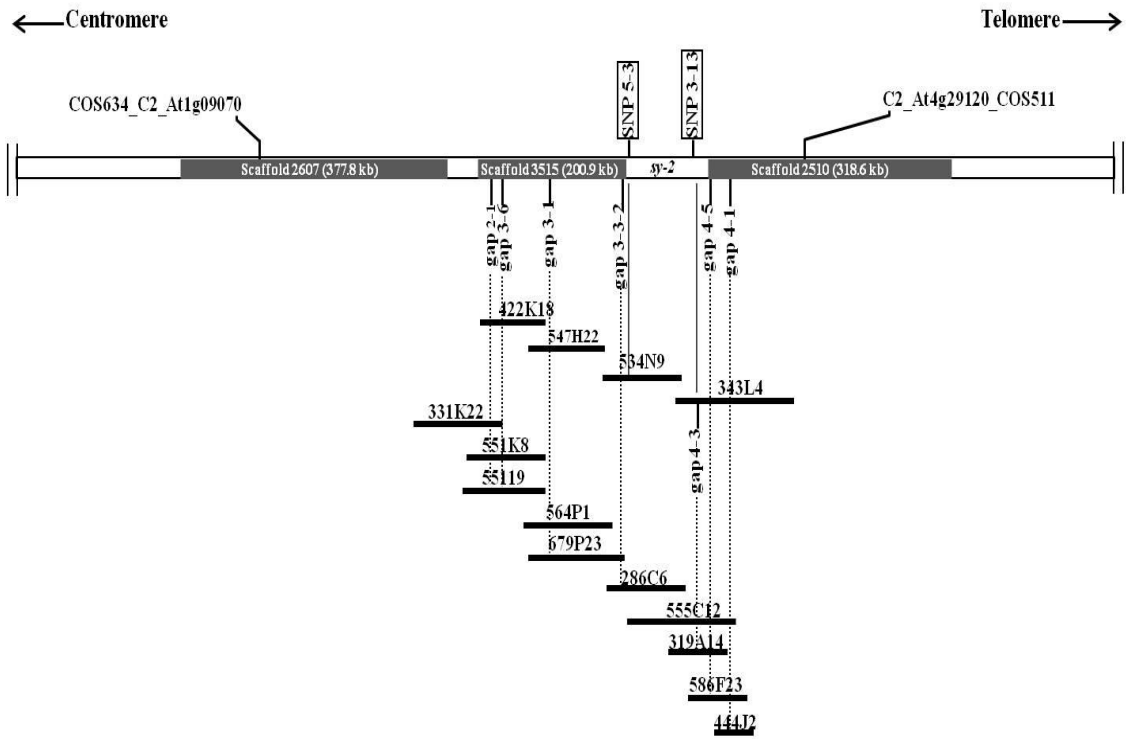
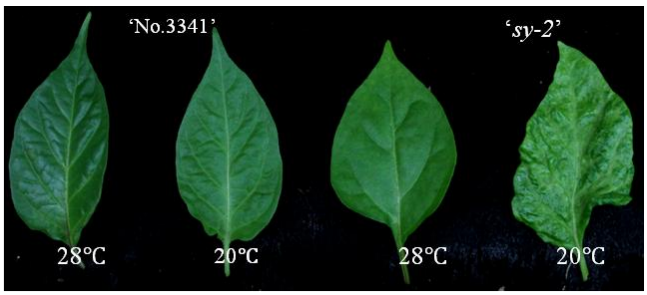


Fig. 1-2. BAC library screening and BAC clones sequence analysis. Seven BAC end markers (gap 2-1, gap 3-6, gap 3-1, gap 3-3-2, gap 4-3, gap 4-5, and gap 4-1) derived from scaffold sequencing were developed for screening a *C. annuum* cv. 'CM334' BAC library (Yoo et al., 2003). Fourteen positive BAC clones were obtained and placed on the *sy-2* locus according to their end sequences. Four BAC clones (422K18, 547H22, 534N9, and 343L4) were completely sequenced to eliminate a gap between scaffolds 3515 and 2510, and two additional SNP markers (SNP 5-3 and SNP 3-13) were identified based on the BAC sequences.

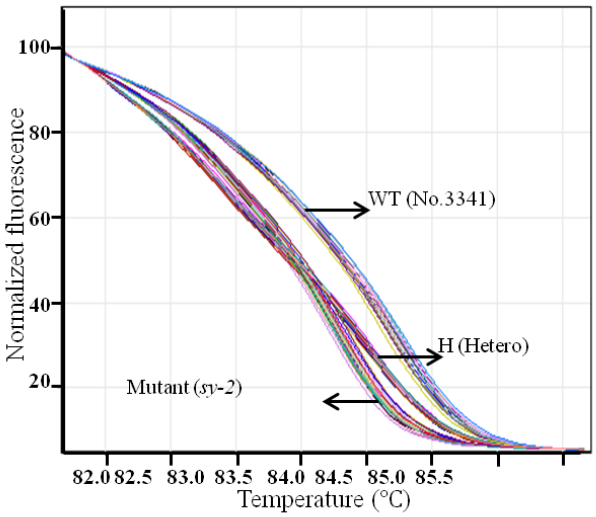
Fine mapping of the *sy-2* locus

To narrow down the target region of the *sy-2* locus, genotype screening was performed using these newly developed markers. A mapping population with 1,433 individuals was used for fine mapping analysis, from which seven recombinant plants (8-2, 10-19, 20-13, 9-35, 1-11, 1-45, and 6-12) were identified; one recombinant for each of KASP 1-2, SNP 5-5, SNP 3-7, and SNP 3-8, and three recombinants for SNP 3-6 (Fig. 1-3). Among the 14 markers used, four SNP markers, SNP 5-3, SNP 3-13, SNP 3-12, and SNP 5-1, were found to be at a genetic distance of 0 cM from the *sy-2* locus. Eight markers (KASP 634, KASP 1-3, KASP 1-2, SNP 2-4, SNP 2-1, SNP 3-6, and SNP 5-5) were located on one end of the *sy-2* gene and two markers (SNP 3-8 and SNP 3-7) were on the other end. The *sy-2* locus was thus delimited to a 0.14 cM region between SNP 5-5 and SNP 3-8 markers on scaffold 3515 and 2510, and SNP 3-12, SNP 5-3, SNP 3-13, and SNP 5-1 markers were located within the *sy-2* locus (Figs. 1-3, 1-4).

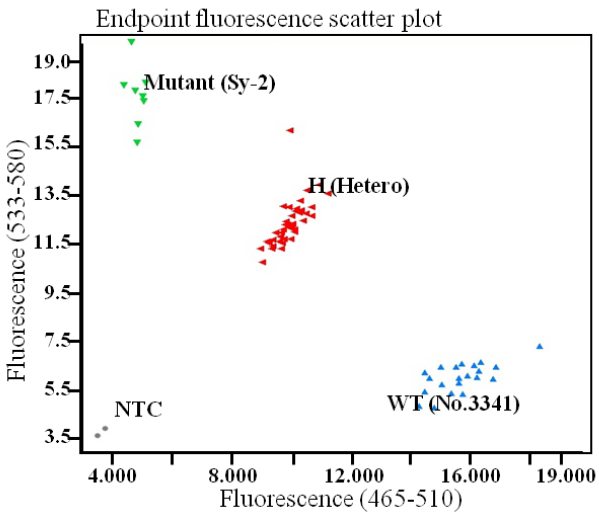
a


'No.3341'
'sy-2'
28°C
20°C
28°C
20°C

b


Normalized fluorescence
100
80
60
40
20
WT (No.3341)
H (Hetero)
Mutant (sy-2)
82.0 82.5 83.0 83.5 84.0 84.5 85.0 85.5
Temperature (°C)

c


Endpoint fluorescence scatter plot
Fluorescence (533-580)
19.0
17.5
15.5
13.5
11.5
9.5
7.5
5.5
3.5
Mutant (Sy-2)
H (Hetero)
NTC
WT (No.3341)
4.000
8.000
12.000
16.000
19.000
Fluorescence (465-510)

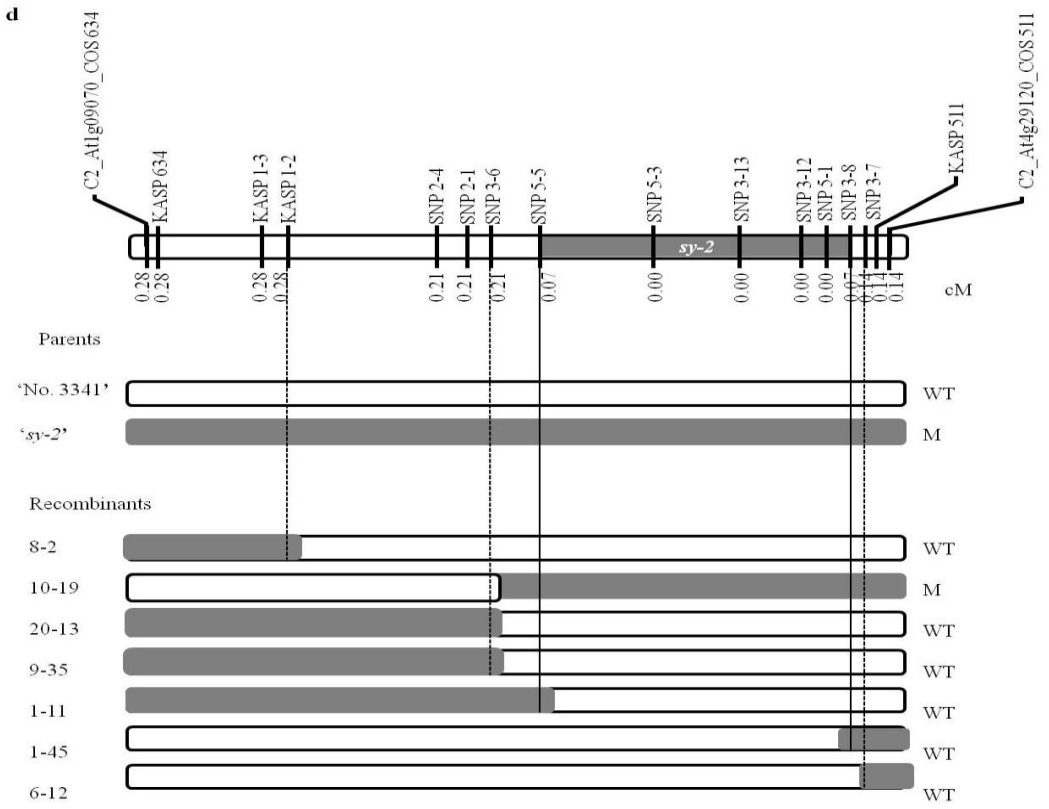


Fig. 1-3. Genotyping and fine mapping of the *sy-2* locus. Genotype screening was performed using a HRM and KASP assays in two F_2 populations. (**a)** Morphological phenotypes observed at the fifth true leaves of *C. chinense* 'No.3341' (WT) and '*sy-2'*(M) at 20 and 28°C. (**b)** Representative HRM analysis identifying three different melting curve types: homozygous dominant genotype (WT), homozygous recessive genotype (M), and heterozygous genotype (H). (**c)** Representative KASP analysis data displayed as a Cartesian cluster plot. Blue data points are homozygous for the allele reported by FAM, red data points are heterozygous, and green data points are homozygous for the allele reported by HEX. The black data points represent no template controls (NTC). (**d)** Fine mapping of the *sy-2* locus. Numbers below the markers indicate genetic distances (cM). White rectangles represent the corresponding fragments of pepper (*C. chinense* 'No.3341') chromosome 1, and black rectangles indicate the *sy-2* chromosome 1. The *sy-2* gene was located between markers SNP 5-5 and SNP 3-8.

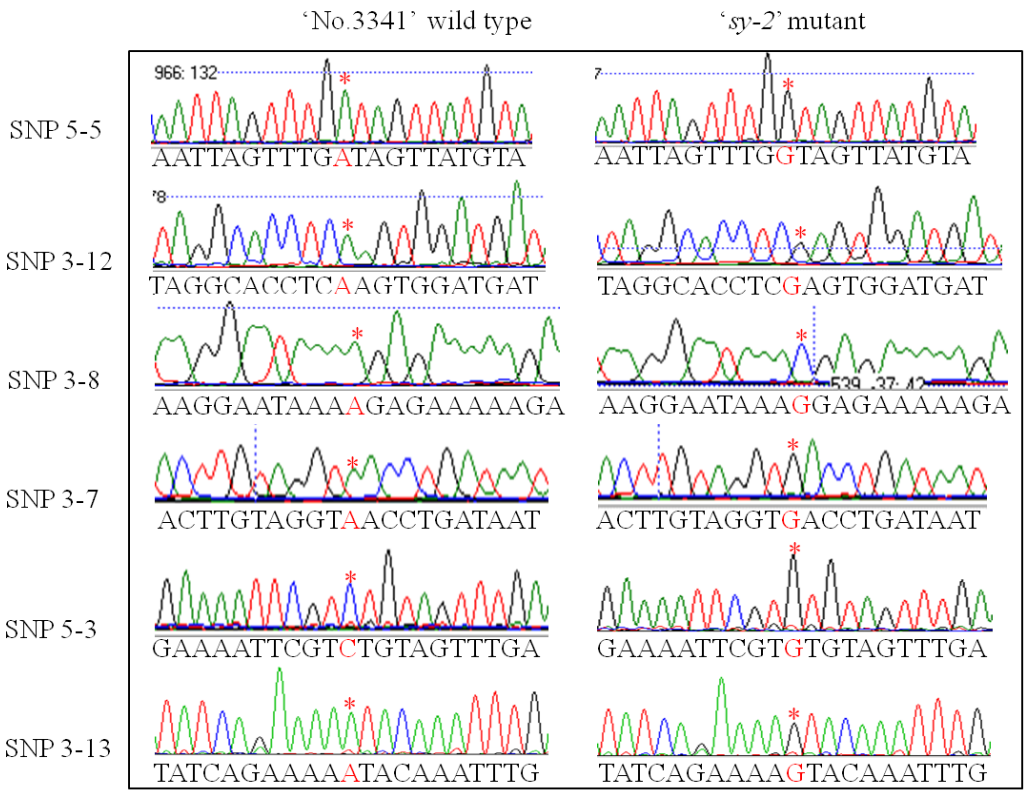


Fig. 1-4. Genomic sequence chromatogram of SNP markers from wild type 'No.3341' and mutant '*sy-2*' plants. Those markers (SNP 5-5, SNP 3-12, SNP 3-8, SNP 3-7, SNP 5-3, and SNP 3-13) were used for genotyping by direct sequencing for fine mapping study. Star indicates the SNPs.

DISCUSSION

In previous studies, 91 COSII markers were used for the genetic mapping of the *sy-2* locus and this was performed using the 148 individuals of F_2 mapping population (An et al., 2011). Among those 91 COSII markers, six flanking markers were tested and found that the *sy-2* is located between C2_At4g29120 and C2_At1g09070. When compared this region with the tomato genome map EXPEN2000 (Wu et al., 2006) and pepper genome sequence database (http://cab.pepper.snu.ac.kr), they found that this region corresponds to the tomato scaffold, scaffold00160, spanning about 300 kb in tomato chromosome 1. Those two COSII markers were identified and located at 3.7 and 4.0 cM, respectively, on chromosome 1 of Tomato-EXPEN 2000 (http//:solgenomics.net).

In the present study, to facilitate identification of the *sy-2* gene, we focused on identifying markers that are more closely linked to the *sy-2* gene through high-resolution genetic mapping of two F_2 populations that consisted of 1,020 and 1,433 individuals. A tomato scaffold sequence (C01HBa0051C14) of 126,295 bp in length, covering the corresponding region of the *sy-2* locus, was identified based on the sequence of the C2_At1g09070 and C2_At4g29120 markers obtained from Sol Genomic Network (SGN) (http//:solgenomics.net). The genotypes and phenotypes were carefully examined for all pepper individuals. A

total of 14 newly developed markers were used, including 4 KASP markers and 10 SNP markers (Figs. 1-1 and Fig. 1-2). We mapped the *sy-2* gene to a 0.14 cM on pepper chromosome 1. The *sy-2* gene was located in an interval of 138.8 kb between markers SNP5-5 and SNP3-8 (Fig. 1-3d).

In this study, the tomato scaffold sequence (C01HBa0051C14) was blasted with the *C. annuum* CM334. V1.5 scaffolds database (updated on January 06, 2014). There were three pepper scaffolds, scaffold 2607 (377.77 kb in size), scaffold 3515 (200.85 kb in size), and scaffold 2510 (318.60 kb in size), which were identified from this database. SNP markers were manually designed in intergenic regions of pepper sequences based on the three non-overlapping pepper scaffold sequences (Fig. 1-1). To identify the target region, we screened 14 positive BAC clones and identified four overlapping clones that cover the gap between the two non-overlapping scaffolds 3515 and 2510 that contain the *sy-2* gene. To narrow down the cloned region containing the *sy-2* gene, we identified four specific SNP markers that co-segregate with the *sy-2* gene with a resolution of 0 cM. The results showed that the *sy-2* gene was located in an interval of 138.8 kb between markers SNP5-5 and SNP3-8 on chromosome 1 (see Figs. 1-3d, 1-4).

The complexity of the pepper genome presents a significant challenge for fine genetic mapping and ultimate map-based cloning of this temperature sensitive gene. In this work we have developed the first high-density linkage map of *sy-2* in

this region, which provides a solid foundation for map-based cloning of the *sy-2* gene.

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CHAPTER II

Map-based Cloning of the Low Temperature Sensitive Gene *sy-2* in Pepper (*Capsicum chinense*)

ABSTRACT

To understand how the '*sy-2*' mutant responds to low temperature, a map-based cloning scheme was used to isolate the *sy-2* gene in pepper (*Capsicum. chinense*). In a previous study, a high-resolution genetic and physical map for the *sy-2* locus was constructed and the *sy-2* gene was fine mapped to a 138.8 kb region between markers SNP 5-5 and SNP 3-8 at the distal portion of chromosome 1, based on comparative genomic analysis and genomic information from pepper. The *sy-2* target region was predicted to contain 27 genes. Based on RT-PCR and RNA-seq gene expression analysis of these predicted genes, *ORF10* and *ORF20* showed a differential expression pattern between mutant and wild-type plants; with both having significantly lower expression in '*sy-2*' than in wild-type plants. In addition, the coding sequences of both *ORF10* and *ORF20* contained single nucleotide polymorphisms (SNPs) causing amino acid changes, which may have important functional consequences. *ORF10* and *ORF20* are predicted to encode F-box proteins, which are components of the SCF complex. Based on the differential expression pattern and the presence of non-synonymous SNPs, we suggest that these two putative F-box genes are most likely responsible for the temperature-sensitive phenotypes in pepper. Further investigation of these

genes may enable a better understanding of the molecular mechanisms of low temperature sensitivity in plants.

INTRODUCTION

Low temperature affects many plant physiological processes, such as water and nutrient uptake, photosynthesis, plant growth, and development (Chinnusamy et al., 2007; Harfied and Prueger, 2015). To understand the molecular basis of low temperature sensitivity in plants, several low temperature-sensitive mutants have been explored. The *Arabidopsis thaliana fatty acid desaturase 2* (*fad2*) mutant deficient in the endoplasmic reticulum (ER) 18:1 desaturase, shows abnormal leaves and a severe dwarf phenotype under low temperature due to the disintegration of the cell membrane (Miquel and Browse, 1994; Zhang et al., 2012). The *Arabidopsis non-photochemical quenching 1* (*npq1)* mutant, which is deficient in xanthophyll cycling, exhibits an accumulation of excessive oxidative stress inhibiting photosystem II (PSII) at low temperature (Havaux and Kloppstech, 2001). The *tobacco temperature dependent shooty* (*tds*) mutant displays abnormal mesophyll cells, thick narrow leaves, and shorter internodes when grown at low temperature (Samuelsen et al., 1997). The *Arabidopsis bonzai1* (*bon1***)** null mutants produce miniature fertile plants, and the *BON1* and *BAP1 (BON1-associated protein)* genes were suggested to have a direct role in regulation of cell expansion and cell division at lower temperatures (Hua et al.,

2001). The mutant maize inbred line *M11* shows remarkably lower accumulation of chlorophyll, which is associated with impaired development and function of chloroplasts (Millerd and McWilliam, 1968). The rice *l*ow *temperature albino 1* (*lta1*) mutant seedlings has albino leaves characterized by remarkably reduced contents of chlorophyll and chlorophyll precursor molecules (Peng et al., 2012). Low temperature response in plants comprises a seemingly disparate set of genetic interactions that make the study of a plant's response to low temperature difficult.

Map-based cloning approaches utilize the relationship between a gene and a marker as the basis for locating a gene. Recently, with the availability of Whole Genome Sequencing (WGS) projects and the development of saturating marker technologies, map-based cloning can now be performed at a higher resolution and the identification of candidate genes in plants is more efficient. In our previous studies, using a pepper mapping population, temperature sensitive gene *sy-2* phenotype was found to be controlled by a single recessive gene and identified several COSII markers closely linked to the gene *sy-2* (An et al., 2011). The complexity of the pepper genome presents a significant challenge for fine genetic mapping and ultimate map-based cloning of this temperature sensitive gene.

Pepper, an important vegetable crop grown worldwide, is temperature-sensitive; its optimum growth temperature lies between 25 and 30°C, and

deviations from these temperatures can adversely affect growth and development, causing a variety of developmental and physiological disorders (An et al., 2011; Onus and Pickersgill, 2004; Koeda et al., 2009, 2010).

In recent years, low temperature sensitive mutants of different plant species have been isolated and characterized to understand the mechanism of low temperature response (Kargiotidou et al., 2008), but the mechanism of low temperature response in pepper is still unclear. We have previously shown a high-density linkage map of the *sy-2* locus on pepper chromosome 1. Based on this previous study, we implemented a map-based cloning approach to identify *sy-2* candidate genes, with the ultimate goal to elucidate the molecular mechanism responsible for the temperature sensitivity of the '*sy-2*'cultivar.

MATERIALS AND METHODS

Plant materials and growth conditions

Two accessions of pepper, *C. chinense* '*sy-2*' and *C. chinense* 'No.3341' were used as parental lines (Koeda et al., 2009). All seeds were sterilized and germinated in an incubator at 30°C in darkness. One week old seedlings were transferred to a growth chamber at 28°C with 16 h light and 8 h dark photoperiod cycle with 60% relative humidity until the cotyledons were fully expanded. The seedlings were then transferred to 20°C chambers, screened for a low temperature-sensitive phenotype after 14 days, and then leaf samples were collected for DNA extraction.

For the RNA-seq analysis, leaf samples from 63 wild-type and 30 mutant phenotype plants from the 2,014 F_2 population were collected for RNA extraction. For RT-PCR and qPCR analyses, leaf samples from '*sy-2*' and 'No.3341' plants grown at 20 and 28°C were used. For virus-induced gene silencing (VIGS) experiments, total 100 wild-type 'No.3341'and 50 '*sy-2*' mutants were used.

RNA extraction and cDNA synthesis

Two to three young leaves from plants of each accession were used for RNA extraction using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was then treated with RNase-free DNase I (Ambion, Austin, Texas, USA) to remove possible contamination of genomic DNA for RT-PCR. Total RNA (2 μg) was used for first-strand cDNA synthesis using M-MLV reverse transcriptase (Invitrogen, Waltham, Massachusetts, USA). The concentration and purity of RNA samples was measured using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA).

RNA-seq library construction

For constructing, RNA-seq library, 63 phenotypical wild-type and 30 phenotypical mutant plants from F_2 populations were exposed to 20°C for 14 days. Total RNA was extracted from young leaves using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentrations and purity were verified for each sample with a Nanodrop 2000 spectrophotometer (Thermo scientific, Waltham, MA, USA). A strand-specific RNA-seq library was constructed as described by Zhong et al., (2011). Briefly, 5 μg total RNA was used for poly(A) RNA enrichment. First-strand and second-strand cDNA was generated using Superscript III reverse transcriptase (Invitrogen). cDNA was purified by AMpure XP beads (Beckman-Coulter, Pasadena, CA, USA).

Following TruSeq adapter ligation, the cDNA was amplified by PCR with 15 reaction cycles. Six random PCR primers were used in this step to obtain short sequence reads. Samples were prepared in three replicates and stored at -20°C.

RNA-seq data analysis

Sample libraries were sequenced using Hiseq 2500 (Illumina/Solexa, San Diego, CA, USA) at NICEM. Sequence reads were aligned to the pepper transcriptome and genome using CLC Genomics Workbench 6.0 (CLC Bio, Prismet, Denmark). The Counts program was used to analyze the number of reads aligning to annotated pepper genes. Expression values in reads per kilobase transcript per million (RPKM) were calculated for the single-map and primary map data sets. Differentially expressed genes were identified using IDEG6 software (Romualdi et al., 2003). Fold-changes are reported as the $\log_2$ of normalized read count abundance for the wild-type samples divided by the read count abundance of the mutants samples. The DESeq tool of the R package (http://www.bioconductor.org) was used to identify differentially expressed genes in wild type 'No.3341' compared with '*sy-2*' with a false discovery rate of <0.05 (Anders et al., 2013).

RT-PCR and qPCR analyses

Total RNA of 2 μg was reverse transcribed to cDNA using an oligo(dT) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). cDNA samples were diluted 4 times for the following test. Gene-specific primers used for RT-PCR and qPCR were designed based on the predicted gene sequences from the target region using a qPCR primer design tool (the Integrated DNA Technology) (Table 2-2). RT-PCR was performed in a total volume of 25 μL containing 4 μL of the diluted cDNA sample, 2.5 μL 10× PCR buffer, 2.0 μL 2.5 mM dNTP mix, 0.5 μL each 10 pmol/μL primer, 0.25 μL Taq polymerase (1 U), and 15.25 μL distilled water. PCR conditions involved initial denaturation for 5 min at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 62°C, and 20 s at 72°C, and a final extension of 5 min at 72°C.

qPCR primers specificity and amplification efficiency were verified by melting curve analysis (after 55 cycles), agarose gel electrophoresis, and sequencing. The qPCR reaction was performed in a Light Cycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) with 20 μL reaction mixture containing 4 μL diluted cDNA sample, 2.0 μL 10× PCR buffer (TaKaRa, Tokyo, Japan), 2.0 μL 2.5 mM dNTP mix, 0.5 μL each 10 μM forward and reverse primer, 0.4 μL rTaq (5 units, TaKaRa), 0.5 μL 50 μM Syto9 and 10.1 μL distilled water. The following PCR cycling conditions were used for qPCR analysis: initial preincubation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C

for 10 s, annealing at 62°C for 10 s and extension at 72°C for 20 s. The pepper *actin* gene was used as the internal control. Relative gene expression levels were calculated using the advanced relative quantification method as implemented with Light Cycler® 480 Real-Time PCR System (Roche).

Gene ontology (GO) term enrichment analysis

Analysis of enriched GO terms was performed for 626 differentially expressed pepper genes with the Blast2GO program using gene annotations downloaded from the NCBI database. Mapping and annotation were performed using default parameters (E-value hit filter of 1.0^{e-6}, annotation cutoff of 55, and GO weight of 5).

Cloning of the *ORF10* and *ORF20* full-length gene sequences

Based on the RNA-seq data and predicted genes *ORF10* and *ORF20* CDS sequences, we designed the primers for cloning *ORF10* and *ORF20* genes (primers details in Table 2-3).

Plasmid construction for VIGS

Tobacco rattle virus (TRV) based VIGS vectors were used in this study. *ORF10* and *ORF20* sequences targeting the 5' UTR of *ORF10* (127 bp) and

ORF20 (127 bp), and *ORF10/20* sequence targeting both *ORF10* and *ORF20* (305 bp) were cloned into a pTRV2 via LIC method (primers details in Table 2-4). pTRV2:*CaPDS* (Chung et al., 2004) was used as control. pTRV2:*CaPDS*, pTRV2:*LIC-5UR-ORF10*, pTRV2: *LIC-5UR-ORF20* and pTRV2:*LIC-ORF10/20* was transformed into *Agrobacterium* strain GV3101 by the freeze-thaw method and VIGS experiment was performed as described (Chung et al., 2004).

***Agrobacterium* infiltration**

Agrobacterium strain GV3101 containing pTRV2 and its derivatives and pTRV1 were cultured in 3 mL Luria-Bertani medium containing 50 mg/L kanamycin and 50 mg/L rifampicin, and shaken-incubated at 28°C overnight. The next day, the *Agrobacterium* cells were collected by centrifugation at 12000 rpm, for 10 min, and suspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone) then the cells' concentration was measured using a NanoDrop (NanoDrop Technologies) in OD_{600} values. The suspension of *Agrobacterium* inocula containing pTRV2 and its derivatives and pTRV1 OD_{600} values were diluted to 1.0. Then the suspension of *Agrobacterium* inocula containing pTRV2 and its derivatives and pTRV1 were mixed at 1:1 volume ratio and incubated for 3 h at room temperature. The *Agrobacterium* was infiltrated into the cotyledons of at two true leaves growth stage by using an 1 mL sterilized syringe without a needle (Liu et al., 2002). The *Agrobacterium*-infiltrated pepper

plants were transferred to 18°C for 1 day and grown in a growth chamber at 20°C under with 16 h light and 8 h dark photoperiod cycle with 60% relative humidity.

Promoter analysis

Promoter analysis was conducted using Softberry software running TSSP (Promoter prediction program for plant genes). Based on the predicted promoter region, we designed the primers for sequencing the promoter region (primers details in Table 2-6). The cloning sequences were analysed by Genomatix software suite (Trial Version): MatInspector: Search for transcription factor binding sites, MatInspector Release professional 8.21, March 2016, MatInspector library: Matrix Family Library Version 9.4 (November 2015), General Core Promoter Elements (0.75/Optimized), Plants (0.75/Optimized).

RESULTS

Prediction of candidate genes

Based on the fine mapping results, the *sy-2* gene was delimited to a 138.8 kb region between the SNP 5-5 and SNP 3-8 markers (Fig. 2-1). Twenty-seven genes (*ORF1* to *ORF27*) were predicted in the target region based on FGENESH analysis, and BLASTP searches carried out at the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that these genes were likely associated with diverse cellular functions (Fig. 2-1, Table 2-1). These predicted genes encoded putative microrchidia (MORC) family proteins (*ORF1*, *ORF4*, and *ORF5*), polyproteins (*ORF2*, *ORF14*, and *ORF17*), cullin-like isoform X1 (*ORF6*), F-box family proteins (*ORF10, ORF20,* and *ORF24*), transposase family protein (*ORF11*), preprotein translocase subunit SecY (*ORF13*), geranylgeranyl pyrophosphate synthase protein (*ORF19*), pentatricopeptide repeat-containing protein (*ORF23*), sucrose nonfermenting 4-like protein-like (*ORF25* and *ORF27*), and several putative uncharacterized proteins (*ORF3*, *ORF7*, *ORF8*, *ORF9*, *ORF12*, *ORF15*, *ORF16*, and *ORF26*). The *sy-2* target region was further analyzed to find homologs of candidate genes from the tomato and pepper genomes. Homologs of *ORF1*, *ORF4*, *ORF5*, *ORF6*, *ORF10*, *ORF11*, *ORF12*, *ORF18*, *ORF19*, *ORF20*, *ORF23*, *ORF24*, *ORF25*, and *ORF27* were identified by

BLAST searches at the Tomato Genome CDS (ITAG release 2.40) and Pepper Annotation CM334 (V1.55) CDS database (Table 2-1).

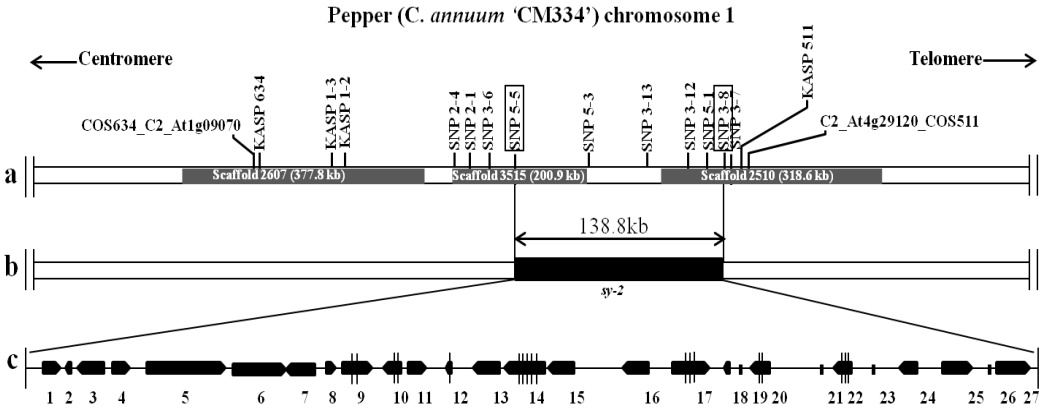

Fig. 2-1. Predicted gene analysis in *sy-2* target region. (**a**) Polymorphism scanning between DNA pools of wild-type ('No.3341') and mutant ('*sy-2*') F_2 generation individuals using SNP markers and KASP markers. (**b**) The target region of gene *sy-2* is 138.8 kb. Open rectangles represent the corresponding fragments of pepper (*C. annuum* 'CM334') chromosome 1, and black rectangles with arrows indicate the target regions. (**c**) Sequence comparison between 'No.3341' and '*sy-2*' on the predicted gene by RNA-seq sequencing analysis.' locations of 27 predicted genes in targeted region.

Table2-1. Genes predicted from the CM334 *sy-2* region using FGENESH program.

Predicted gene	Protein size (aa)	Putative SNP*	NCBI BlastP hit	Species	E value	Identity (%)	GenBank ID
ORF1	223	-	PREDICTED: uncharacterized protein LOC102603698	*Solanum tuberosum*	1.00E-85	91	XP_006344837.1
			PREDICTED: MORC family CW-type zinc finger protein 3-like	*S. lycopersicum*	4.00E-84	89	XP_004230214.1
				Nicotiana sylvestris	1.00E-83	87	XP_009791922.1
ORF2	288	-	PREDICTED: uncharacterized protein LOC104109376	*N. tomentosiformis*	7.00E-138	65	XP_009616949.1
			Putative polyprotein (reverse transcriptase)	*S. demissum*	9.00E-137	65	AAT40504.2
			PREDICTED: uncharacterized protein LOC103417687	*Malus domestica*	1.00E-106	54	XP_008354068.1
ORF3	581	-	PREDICTED: uncharacterized protein LOC104090989	*N. tomentosiformis*	4.00E-171	57	XP_009594512.1
			PREDICTED: uncharacterized protein LOC104213818	*N. sylvestris*	5.00E-154	53	XP_009761666.1
			T4.15	*Malus x robusta*	1.00E-148	47	CCH50976.1
ORF4	114	-	PREDICTED: MORC family CW-type zinc finger protein 3-like	*S. lycopersicum*	2.00E-33	85	XP_004230214.1
			PREDICTED: uncharacterized protein LOC102603698	*S. tuberosum*	1.00E-32	85	XP_006344837.1
			PREDICTED: MORC family CW-type zinc finger protein 3 isoform X2	*N. sylvestris*	6.00E-31	81	XP_009791922.1
ORF5	708	-	PREDICTED: uncharacterized protein LOC102603698	*S. tuberosum*	0	73	XP_006344837.1
			PREDICTED: MORC family CW-type zinc finger protein 3-like isoform X1	*N. tomentosiformis*	2.00E-178	67	XP_009605484.1
				N. sylvestris	4.00E-173	66	XP_009791921.1
ORF6	613	-	PREDICTED: cullin-1-like isoform X1	*S. tuberosum*	5.00E-138	77	XP_006344634.1
			PREDICTED: cullin-1 isoform X1	*S. lycopersicum*	4.00E-137	76	XP_004230212.1

			PREDICTED: cullin-1-like isoform X1	*N. sylvestris*	3.00E-131	73	XP_009791917.1
ORF7	246	-	Hypothetical protein VITISV_041110	*Vitis vinifera*	1.00E-13	38	CAN67668.1
			PREDICTED: uncharacterized protein LOC104096858	*N. tomentosiformis*	1.00E-12	39	XP_009601594.1
			PREDICTED: uncharacterized protein LOC104220982	*N. sylvestris*	4.00E-11	38	XP_009770256.1
ORF8	328	-	PREDICTED: uncharacterized protein LOC102598771	*S. tuberosum*	1.00E-69	55	XP_006340519.1
			PREDICTED: uncharacterized protein LOC104879080	*V. vinifera*	1.00E-59	48	XP_010648881.1
			PREDICTED: uncharacterized protein LOC104882691	*V. vinifera*	5.00E-59	48	XP_010665180.1
ORF9	486	T935C (E), A952G (I/T)	Hypothetical protein VITISV_013540	*V. vinifera*	6.00E-159	44	CAN74029.1
			Hypothetical protein VITISV_033829	*V. vinifera*	1.00E-157	46	CAN63433.1
			Hypothetical protein VITISV_043230	*V. vinifera*	1.00E-156	47	CAN64779.1
ORF10	450	T691G (C/G), A943G (N/D)	PREDICTED: F-box protein At5g49610-like	*N. sylvestris*	1.00E-92	45	XP_009800259.1
			PREDICTED: F-box/kelch-repeat protein At3g23880-like	*N. sylvestris*	1.00E-87	44	XP_009774243.1
			PREDICTED: F-box/kelch-repeat protein At3g06240-like	*S. tuberosum*	4.00E-78	40	XP_006344952.1
ORF11	436	NA	PREDICTED: uncharacterized protein LOC104099247	*N. tomentosiformis*	1.00E-69	33	XP_009604477.1
			Putative transposase, identical	*S. tuberosum*	1.00E-68	41	AAV31178.1
			PREDICTED: uncharacterized protein LOC104243264	*N. sylvestris*	1.00E-67	36	XP_009796727.1
ORF12	295	T267G (D/E)	PREDICTED: uncharacterized protein LOC104112146	*N. tomentosiformis*	5.00E-30	37	XP_009620284.1
			PREDICTED: uncharacterized protein LOC104229227	*N. sylvestris*	7.00E-26	40	XP_009780137.1
			PREDICTED: uncharacterized protein LOC104092591	*N. tomentosiformis*	2.00E-23	31	XP_009596518.1
ORF13	202	-	Preprotein translocase subunit SecY	*Brevibacillus borstelensis*	6.9	30	WP_003391941.1
ORF14	1329	A122G (N/S), G125A (R/Q), T378C (H),	PREDICTED: uncharacterized protein LOC104648490	*S. lycopersicum*	0	69	XP_010324108.1
			PREDICTED: LOW QUALITY PROTEIN: uncharacterized protein LOC104108577	*N. tomentosiformis*	0	62	XP_009615944.1

		T2205C (F), G3163A (Q/R)	Putative polyprotein	*S. demissum*	0	64	AAT39963.2
ORF15	340	-	PREDICTED: uncharacterized protein LOC104109516	*N. tomentosiformis*	6.00E-64	42	XP_009617137.1
			PREDICTED: uncharacterized protein LOC104111769	*N. tomentosiformis*	5.00E-63	43	XP_009619833.1
			PREDICTED: uncharacterized protein LOC104087707	*N. tomentosiformis*	2.00E-62	54	XP_009590557.1
ORF16	285	-	Hypothetical protein PRUPE_ppa025708mg	*Prunus persica*	1.00E-29	38	XP_007198912.1
			PREDICTED: uncharacterized protein LOC104648891	*S. lycopersicum*	7.00E-28	32	XP_010325234.1
			PREDICTED: uncharacterized protein LOC102587225	*S. tuberosum*	2.00E-27	36	XP_006341875.1
ORF17	522	A537G (P), C546T (F), A547G (I/V)	Hypothetical protein VITISV_003451	*V. vinifera*	9.00E-90	51	CAN73567.1
			Putative polyprotein	*S. demissum*	7.00E-85	77	ABI34306.1
			Putative gag and pol polyprotein, identical	*S. demissum*	2.00E-84	75	AAU90333.1
ORF18	108	-	No hits	-	-	-	-
ORF19	67	-	Geranyl geranyl pyrophosphate synthase	*N.langsdorffiixN. sanderae*	9.00E-27	80	ABB29853.1
			Chloroplast geranylgeranyl diphosphate synthase 3	*N. tabacum*	2.00E-25	82	AFB35651.1
			Geranylgeranyl pyrophosphate synthase 3	*S. pennellii*	3.00E-25	82	ADZ24720.1
ORF20	467	G767A and T768A (S/K)	PREDICTED: F-box/kelch-repeat protein At3g23880-like	*N. sylvestris*	3.00E-96	43	XP_009774243.1
			PREDICTED: F-box protein At5g49610-like	*N. sylvestris*	9.00E-96	45	XP_009800259.1
			PREDICTED: F-box/kelch-repeat protein At3g06240-like	*N. tomentosiformis*	2.00E-78	41	XP_009622437.1
ORF21	56	-	No hits	-	-	-	-
ORF22	452	A655G (K/E), T856G (Y/D), G1144T (M/I)	No hits	-	-	-	-

ORF23	81	-	PREDICTED: putative pentatricopeptide repeat-containing protein At5g06400, mitochondrial	*S. lycopersicum*	6.00E-08	51%	XP_004228915.2
			PREDICTED: putative pentatricopeptide repeat-containing protein At5g06400, mitochondrial-like	*S. tuberosum*	2.00E-06	51%	XP_006348568.1
			Hypothetical protein DAPPUDRAFT_267284	*Daphnia pulex*	0.38	33%	EFX63986.1
ORF24	153	-	PREDICTED: F-box/LRR-repeat protein At3g03360-like	*N. tomentosiformis*	1.00E-07	57%	XP_009618684.1
			PREDICTED: probable F-box protein At1g60180	*N. sylvestris*	2.00E-06	44%	XP_009794809.1
			PREDICTED: F-box/LRR-repeat protein 25-like	*N. tomentosiformis*	1.00E-05	47%	XP_009611668.1
ORF25	345	-	PREDICTED: sucrose nonfermenting 4-like protein-like	*S. tuberosum*	1.00E-125	71%	XP_006344633.1
				N. tomentosiformis	5.00E-125	69%	XP_009588179.1
				N. sylvestris	4.00E-124	68%	XP_009791916.1
ORF26	114	-	Hypothetical protein	*Pseudomonas aeruginosa*	0.63	27%	WP_023121221.1
				P. aeruginosa	1.2	30%	WP_042913417.1
				P. aeruginosa	1.2	30%	WP_031642169.1
ORF27	327	-	PREDICTED: sucrose nonfermenting 4-like protein	*S. lycopersicum*	2.00E-145	95%	XP_004230211.1
				S. tuberosum	2.00E-144	94%	XP_006344633.1
				N. sylvestris	1.00E-139	91%	XP_009791916.1

* SNP positions are indicated relative to the initiation codon of predicted gene sequences from CM334.

The SCF complex, which is a multi-protein E3 ubiquitin ligase complex formed by four major components: an S-phase kinase-associated protein 1 (SKP1), Cullin 1 (CUL1), RING-box 1 (RBX1), and an F-box protein, mediates the ubiquitination of proteins destined for proteasomal degradation (Jain et al., 2007; Lyzenga and Stone 2012; Schumann et al., 2011; Vierstra 2003). Interestingly, four putative genes, *ORF6* (encoding cullin-like isoform X1), *ORF10*, *ORF20*, and *ORF24* (encoding F-box proteins) related to the SCF complex were identified within the *sy-2* locus. *ORF10* and *ORF20* shared 93.4 and 90.0% nucleotide and amino acid sequence identity, respectively. The *ORF24* (F-box/LRR-like protein) shared no significant sequence similarity with either *ORF10* or *ORF20*. F-box proteins contain a highly conserved approximately 50-amino acid F-box motif at their N-terminus, and C-terminal protein-protein interaction domains, such as kelch repeats, DEAD box, leucine-rich repeats, WD40 repeats, or Armadillo (Jain et al., 2007; Schumann et al., 2011; Vierstra 2003). The predicted protein sequences of *ORF10* and *ORF20* showed putative C-terminal kelch repeats containing domain in addition to the N-terminal F-box domain, whereas *ORF24* likely encoded a truncated F-box protein as it was lacking the F-box domain (Fig. 2-2).

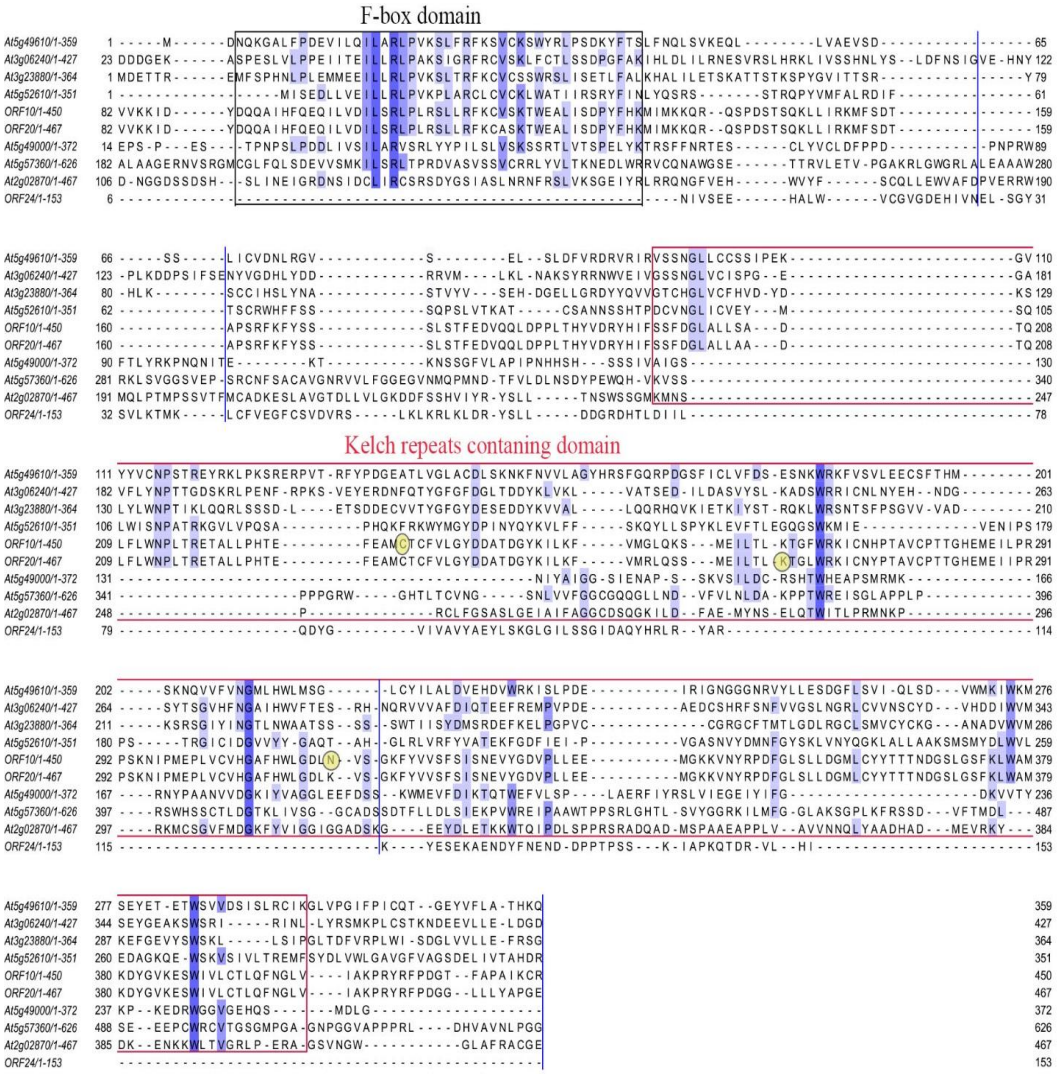

Fig. 2-2. Multiple sequence alignment of the predicted pepper F-box proteins with the *Arabidopsis* F-box/kelch-like protein sequences. The predicted F-box domain and kelch repeats-containing domains are boxed. The '*sy-2*' mutated amino acid positions in *ORF10* and *ORF20* are indicated with circles.

Sequence variations of predicted genes

Putative SNP sites were identified by comparing the coding sequences from wild-type and mutant plants. Sequence alignment showed two SNPs each in *ORF9*, *ORF10*, and *ORF20*, one SNP in *ORF12*, five SNPs in *ORF14*, and three SNPs each in *ORF17* and *ORF22* (Table 2-1). In *ORF9*, out of the two SNPs identified (T935C and A952G), only SNP A952G resulted in an amino acid change (I318T). In *ORF10*, both SNPs, T691C and A943G, caused amino acid changes, C231G and N315D, respectively. In *ORF20*, two consecutive SNPs, G767A and T768A caused an amino acid change, S256K. In *ORF12*, the T267G SNP caused an amino acid change, D89E. Out of five SNPs identified in *ORF14* (A122G, G125A, T378C, T2205C, and G3163A), three SNPs A122G, G125A, and G3163A caused amino acid changes, N41S, R42Q, and Q1055R, respectively. Out of the three SNPs identified in *ORF17* (A537G, C546T, and A547G), SNP A547G resulted in an amino acid change, I183V. The three SNPs in *ORF22* (A655G, T856G, and G1144T) resulted in amino acid changes K219E, Y286D, and M382I, respectively (Table 2-1).

Expression analysis of predicted genes

RT-PCR was carried out to identify which candidate genes showed a differential expression pattern between wild-type and mutant plants grown at two

different temperatures 20 and 28°C. Several predicted genes from the *sy-2* target region, *ORF1*, *ORF2*, *ORF3*, *ORF4*, *ORF5*, *ORF6*, *ORF7*, *ORF13*, *ORF15*, *ORF18*, *ORF19*, *ORF21*, *ORF22*, *ORF24*, *ORF25*, and *ORF27*, showed no expression differences between the wild-type and '*sy-2*' plants (Fig. 2-3). Weak, but detectable expression was observed in the case of *ORF11*, *ORF12*, and *ORF23* in both the wild-type and '*sy-2*'plants. *ORF9*, *ORF10*, and *ORF26* were down-regulated in both wild-type and '*sy-2*' plants at low temperature. However, the expression level of *ORF10* was much higher in wild-type plants than in '*sy-2*' plants in both conditions. Several genes, *ORF8*, *ORF14*, *ORF17*, and *ORF20*, were observed to be up-regulated in both wild-type and '*sy-2*' plants under cold stress, although the expression of *ORF20* was higher in wild-type plants than '*sy-2*' plants.

Table 2-2. RT-PCR/qPCR primers used in the experiment.

Primer	Sequence	Amplicon (bp)
ORF1-RT	F: GTTGTGCTTTCAGTTACCGTC	127
	R: TTTGCAGAACTCATCCCAGG	
ORF2-RT	F: AAAACTAAGGTGAGGACGGC	135
	R: CTCCCCTTGAATACGCCAC	
ORF3-RT	F: CTCTTCCCCTCAACTATACAGC	104
	R: TCCTCTGCGATAGGTATGTCG	
ORF4-RT	F: GGTGAACCTTTCTTTGGCAATAA	109
	R: GGATACTGGAACAGCTTAAAGGA	
ORF5-RT	F: ACCACATCTGACCACAATGG	150
	R: CAGTTTGGTGCAGAACAGATTC	
ORF6-RT	F: TCTTCCAAGGGACCCATTTTC	134
	R: CGTCATTCACAGCTTCTCAAAG	
ORF7-RT	F: AGGTGGGAACTGAAACTTGG	142
	R: AATGACCTCAGCCCAATCTG	
ORF8-RT	F: GCCCTTTTCTTGGATCTGTTTG	146
	R: CGACATTCCACAAGTTTAGAGC	
ORF9-RT	F: GGTGATGGAGATGGTGAGATTC	121
	R: GTCTTGGAGTGCTCTAGTTGG	
ORF10-RT	F: TCCAGATGGTGAGCTGCTACTATTTT	86
	R: TGGATGTCTGGAATGTAAAACAGCTAAC	
ORF11-RT	F: GTAATACCCATAAGTAGCCACTCG	148
	R: GGGAAAGGAATTTGTGATTGAAAAG	
ORF12-RT	F: CTCTACAAGTGTTGCATTGGTG	139
	R: TCTTATTCTGCGAGGGACAAC	
*ORF1*3-RT	F: AAGGAACAGCCCAGGAAAG	132
	R: TTTACCATGCCCCTGAGATG	
ORF14-RT	F: AGGAGAAATCGGTGAATGTAGC	123
	R: TTCTGGTGCCTTGTACTGTG	
ORF15-RT	F: TGCTGTGGTTCTCTGTTTCTC	105
	R: TGCTACGGGATGTGATTTGG	
ORF16-RT	F: TTTCCGCCATGTGGTTAGAG	145
	R: AGCATAACACTGACCACGAC	
ORF17-RT	F: CCACCTAGCTCAGTTGCATT	106

	R: TAGGAGTGAGAGCCGTTATGA	
ORF18-RT	F: AGGTGATGGAAATAGGGTTATGG	110
	R: AATAGTATATCACCATATCCACCTTCC	
ORF19-RT	F: CTGGGAAGGACTTAGTTGCTG	120
	R: GATCAAATCCAGCAAGCTTAGC	
ORF20-RT	F: TCCAGATGGTGGGCTGCTACTATATG	86
	R: TGAATGTCTGGAATGTAAAACAGCTAGG	
ORF21-RT	F: CTTCCGGCATATGACTTGAATTATC	145
	R: TGCACAAAGATGAATTCCTCAAAG	
ORF22-RT	F: GCCTTGATGGCTCGGAATAA	119
	R: CTCCTCAAAGCATCCACACTAC	
ORF23-RT	F: TCTCGTATCGAACCTGTTTGTG	125
	R: ACATCCCTTGTATCTTCGCG	
ORF24-RT	F: ACAATATTTGACACATTCGAGCG	134
	R: AAATATTCATTTCTGTTCTTTAGTGATGC	
ORF25-RT	F: TGTTTGGGTCTGGAAGTGATAG	132
	R: CCTGACTAGTGAAAGAGCCAC	
ORF26-RT	F: AGAAAACATAGCAGGCGCTCCATAA	120
	R: GGCTAAACTCCTTCAAGCCTTTGAAACTAT	
ORF27-RT	F: TCATTTCCTCAGCTGCTACAC	124
	R: CGGCAGATACCTGGACATTT	
CaActin	F: AGGAATGGTTAAGGCTGGATTT	200
	R: TCTTCTCCATATCGTCCCAGTT	

F, forward; R, reverse.

Based on these results, next qPCR was performed to investigate further gene expression patterns of *ORF10* and *ORF20* (Fig. 2-4). Consistently with our RT-PCR results, *ORF10* and *ORF*20 showed differential expression between wild-type and mutant plants. Expression levels of *ORF10* were 8.2 times higher in wild-type plants than in '*sy-2*' plants at 20°C. Similarly, expression levels of *ORF10* were 8.9 times higher in wild-type plants than in '*sy-2*' plants at 28°C. Expression of *ORF20* was 1.2 and 1.8 times higher in wild-type plants compared with '*sy-2*' plants at 20 and 28°C, respectively. At low temperature, *ORF10* was observed to be slightly down-regulated, whereas *ORF20* was significantly up-regulated, suggesting differential expression patterns under cold stress.

RNA-seq analysis was carried out to further understand the pepper transcriptome change in response to low temperature. We have mapped and quantified the transcriptome by deeply sequencing pepper RNA and recording how frequently each gene is represented in the sequence sample. Based on genotype and phenotype analysis results, a total of 93 F_2 breeding lines (60 wild-type and 33 mutants) treated with low temperature (20°C) were used to construct six RNA-seq libraries (Wild-type rep 1, 2, 3 and *sy-2* mutant rep 1, 2, 3). All twenty-seven predicted ORFs were used as reference sequences to construct a heat map of the differential expression of predicted ORFs between wild-type and '*sy-2*' mutant under cold stress condition. Seventeen predicted ORFs were tested

in this analysis (Fig. 2-5). The result showed that *ORF10* and *ORF20* were observed to be down-regulated; *ORF8, ORF9,* and *ORF19* were observed to be up-regulated in both wild-type and '*sy-2*' plants at low temperature. The expression of *ORF7, ORF9,* and *ORF17* were weak, and the expression of *ORF2, ORF3, ORF4, ORF6,* and *ORF27* were not observed to change in the heat map (Fig. 2-5).

Among the predicted *sy-2* candidate genes, the *ORF10* and *ORF20* genes, which belong to the Kelch type F-box genes, were observed to be differentially expressed in wild-type and '*sy-2*' plants. Expression levels of *ORF10* and *ORF20* were significantly higher in wild-type plants. Furthermore, nonsynonymous mutations in the *ORF10* and *ORF20* could affect their posttranslational modification and protein-protein interactions. F-box proteins, as a component of the *SCF* E3 ubiquitin ligase, play an important role in conferring substrate specificity to the SCF complex (Jain et al., 2007; Schumann et al., 2011; Skaar et al., 2013; Vierstra, 2003). As such, F-box proteins play crucial roles in regulating various plant developmental and stress responses by integrating nearly all hormonal signaling pathways (Dreher and Callis, 2007; Kim et al., 2013; Li et al., 2016; Panstruga et al., 2009; Shabek and Zhang 2014; Vierstra, 2003). These observations led us to propose that the F-box genes, *ORF10* and *ORF20,* are likely *sy-2* candidate genes, and differences in their expression levels and

presence of nonsynonymous mutations could be responsible for the temperature sensitivity in '*sy-2*' plants.

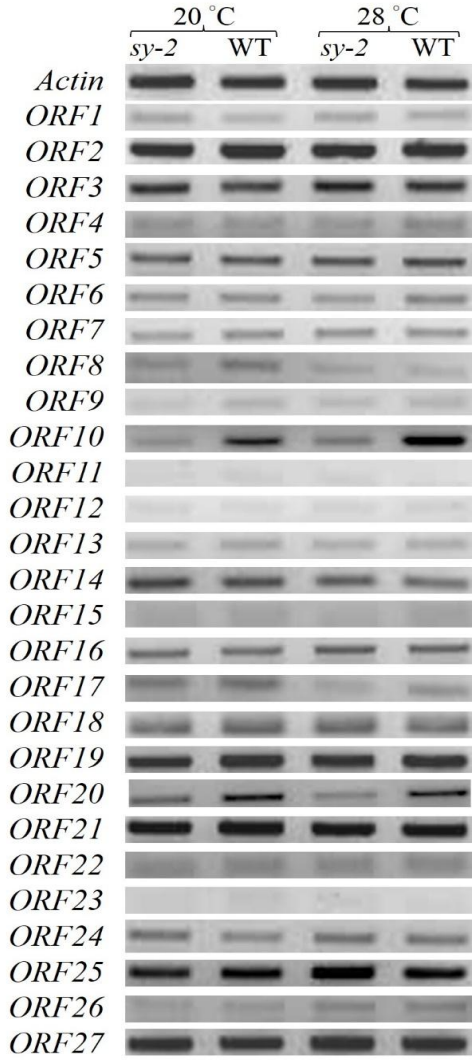

Fig. 2-3. RT-PCR analysis of predicted *sy-2* genes from 'No.3341' and '*sy-2*' plants at 20 and 28°C.

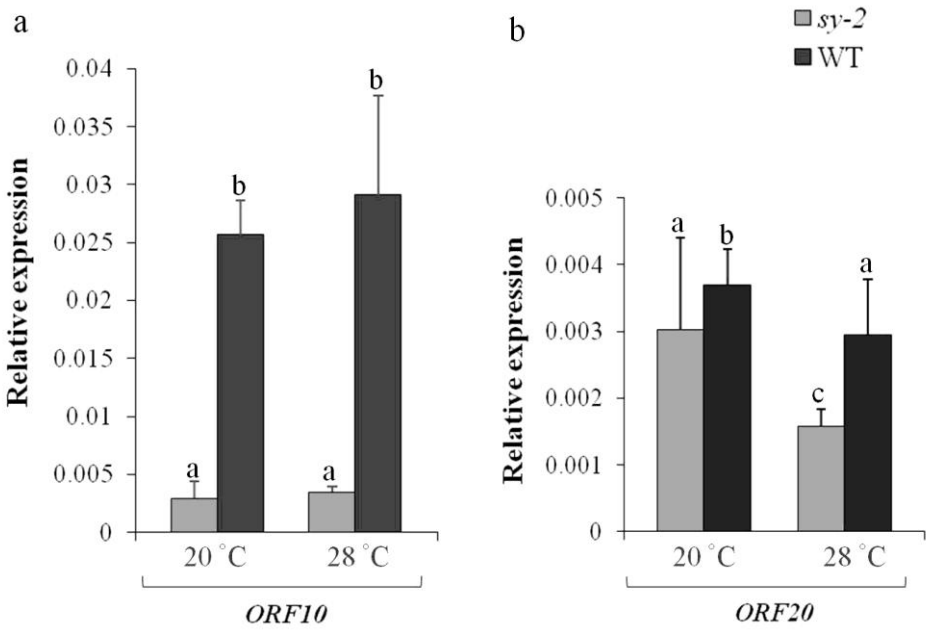

Fig. 2-4. qPCR analysis of predicted genes from 'No.3341' and '*sy-2*' plants at 20 and 28°C. *Actin* was used as an internal control. Different letters indicate significant differences within (**a**) the groups *ORF10* and (**b**) *ORF20* according to Duncan's multiple range test at $p \leq 0.05$.

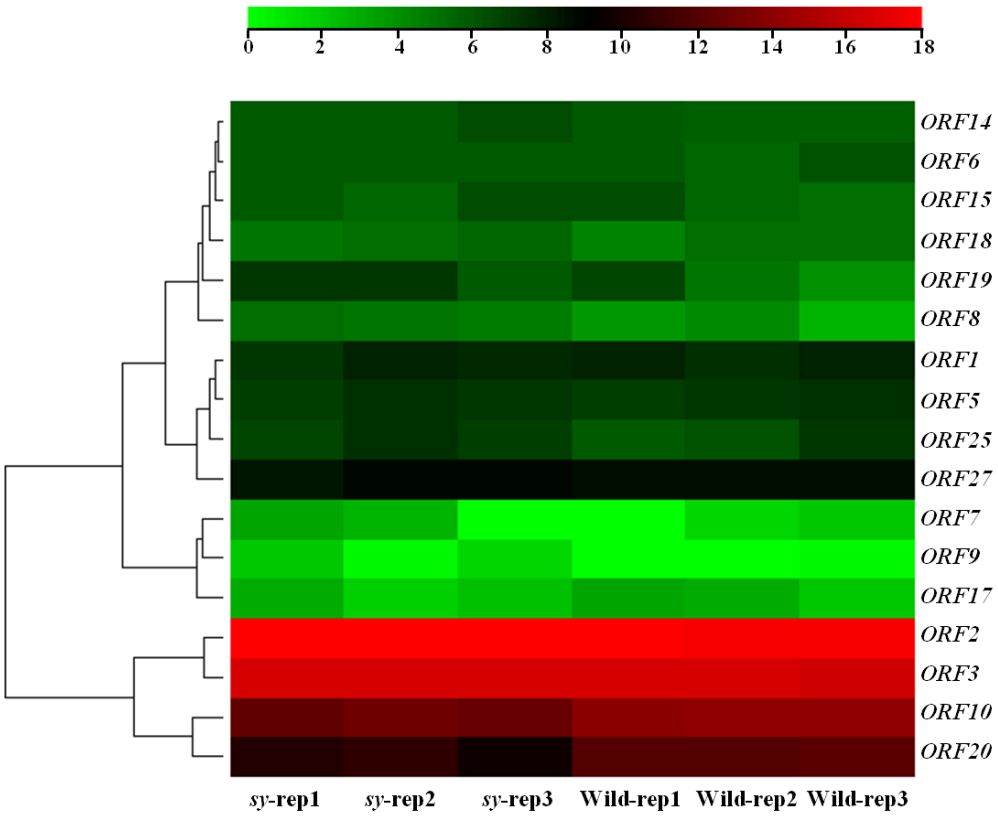

Fig. 2-5. Heatmap generated from the 27 differentially expressed genes as reported by R console (version 3.2.2). Genes are according to genes' RPKM change (log2). The green color indicates positive regulates of gene expression while red indicates negative regulate expression.

GO term enrichment analysis

To identify potentially altered biological processes under low temperature in *Capsicum*, the top 626 differentially expressed genes identified by RNA-seq were used for GO term enrichment analysis (Fig. 2-6). These results showed that, in the biological process category, cellular process, response to stimulus, single-organism process, and response to stress were the most highly represented groups, suggesting that major metabolic changes take place to maintain tissue activity in low temperature. In the cellular component category, transcripts that correspond to the cell, cell parts, intracellular, and intracellular part were typically the most enriched. This suggests that low temperature affects cellular components. Binding and catalytic activities were the two groups most highly enriched within the molecular function category, suggesting that posttranslational modifications might be involved in the regulation of expression of at least some of the stress-responsive genes to cope with low temperature.

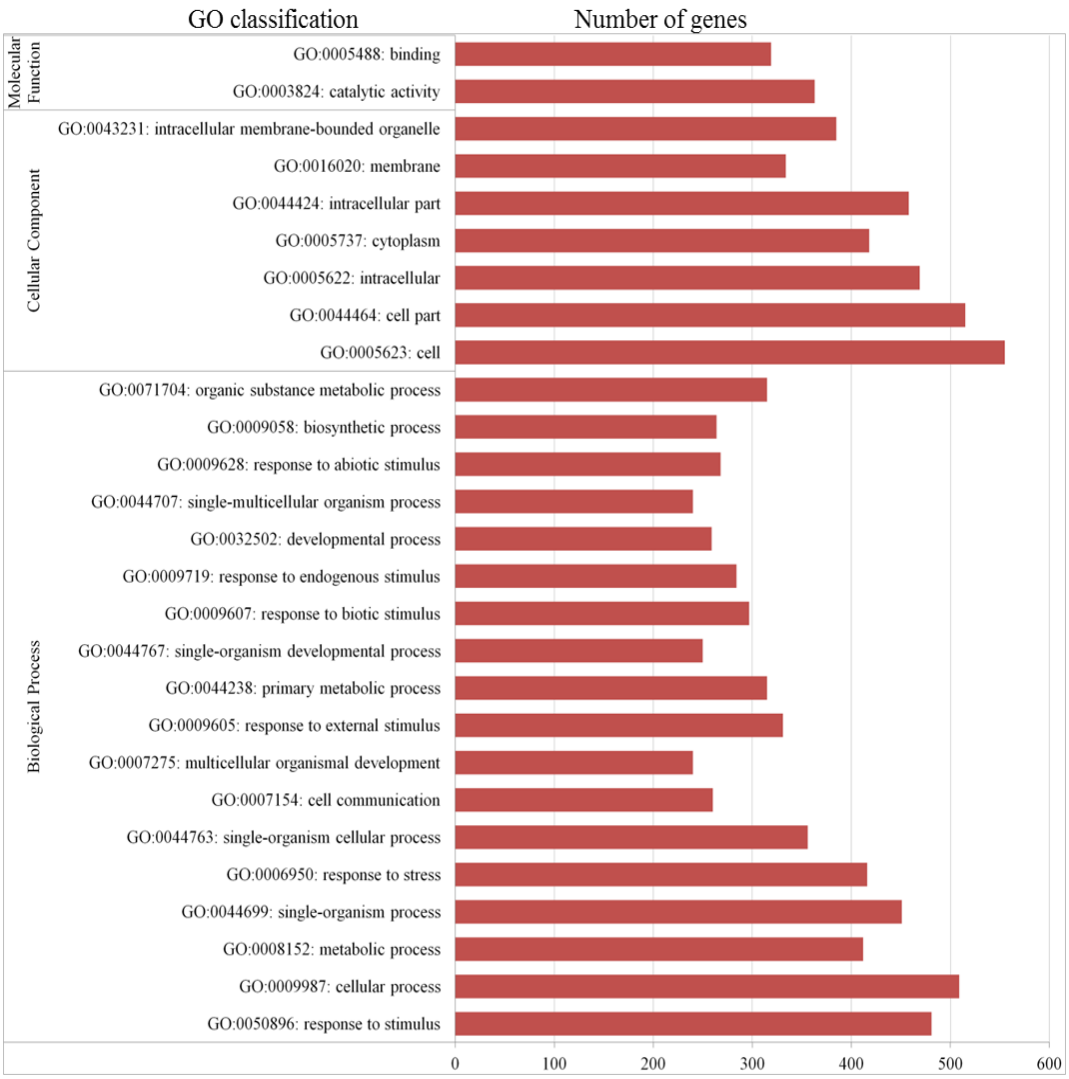

Fig. 2-6. Gene ontology (GO) term analysis of differentially expressed genes of *Capsicum* at 20°C. GO analysis was carried out using the Blast2 go program. The GO terms were grouped into different categories at level 2.

Table 2-3. Primer sequences used for cloning of pepper *ORF10* and *ORF20* full-length cDNAs.

Primer	Sequence	Amplicon (bp)
*ORF10*UtVig	F: AGGTGT TATTTTTGTCCT TTCCCCTAA	1904
ORF10-TAA	R: TTAGATAACTAATTTTGGAGAAATCAAGCTTTCT	
*ORF20*UtVig	F: GAAAAATGATTTTATGATATCTAACTTGT	1966
ORF20-TAG	R: CTAAATAACTAATTTTGGAGAAATCAAGCTTTCT	

F, forward; R, reverse.

Table 2-4. Primers used for VIGS.

Primer	Sequence
LIC-*ORF10*Utvig	F: CGACGACAAGACCCTAGGTGTTATTTTTGTCCTTTCC
	R: GAGGAGAAGAGCCCTCTGCAAACTCACTGTACAATTTAG
LIC-*ORF20*Utvig	F: CGACGACAAGACCCTGAAAAATGATTTTATGATATC
	R: GAGGAGAAGAGCCCTCAATGTATAATTTAGTCCACTGAAAAT
LIC-*ORF10/20*Utvig	F: CGACGACAAGACCCTTCCAAAACTTGGGAG
	R: GAGGAGAAGAGCCCTGCGGTTTCTCTTGTTAAGGGGT
pPDS	F: GCAATGGAAGGAACATTCGA
	R: AGTCTCTCAGGAGGATTACC

F, forward; R, reverse.

VIGS analysis in 'No.3341' plants

To characterize the function of two F-box candidate genes, gene silencing by VIGS approach was utilized. For this study, wild-type 'No.3341' was used as test plants. Fragments of *CaPDS* (420 bp), *ORF10* (127 bp), *ORF20* (127 bp), and *ORF10/20* (305 bp) were transformed into *Agrobacterium* strain GV3101 by the freeze-thaw method (Fig. 2-7).

The photobleaching phenotype of *PDS* silencing in leaves by pTRV2:*CaPDS* was observed at 3 weeks after inoculation (Fig. 2-8). All silenced plants resulted in a dwarf phenotype. *ORF10*-silenced pepper plants showed light yellow leaves and slightly abnormal phenotype shape, as well as moderate growth retardation. Suppression of the *ORF20* gene results in a rough surface and pale-green parts. To observe double silencing the *ORF10* and *ORF20* genes, C was inoculated in silenced pepper plants. Silenced plants produced small, asymmetric, and thicker true leaves, with loss of pigmentation (Fig. 2-8, Table 2-5).

To confirm that the phenotype changes in leaf involve VIGS of the two candidate genes, up- and down-regulations of the mRNA levels of this two F-box genes were analyzed by RT-PCR and qPCR (Figs. 2-9, 2-10). The mRNA levels of the genes in silenced plants were measured by RT-PCR with different cycles (18, 22, 26, 30 cycles). The gene expression of *ORF10* and *ORF20* were not showed significantly decreased in *ORF10*-silenced pepper plants and *ORF20*-

silenced pepper plants. But the *ORF10* and *ORF20* were showed significantly decreased in double silencing pTRV2:*LIC-ORF10/20* gene plants (Fig.2-9).

Quantification of the signal intensity of qPCR comparing to *CaActin* gene as an internal control indicated that the double silenced ORF10/20 gene mRNA levels were reduced compared to the levels in control MOCK plants. There is no significant change in expression of MOCK (TRV2:*00*) and ORF10 silenced VIGS plants. Compared with MOCK plant, ORF10/20 silenced plants showed almost 4.8 times down-regulated expression of ORF10. There is no significant change in expression of MOCK and ORF20 silenced VIGS plants. Compared with MOCK, ORF10/20 silenced plants showed almost 4.8 times downregulated expression of ORF20 (Fig. 2-10).

The recovery experiment was performed to determine if the growth induced phenotypes observed at 20°C in the double mutant plants was reversible. 3 double silenced pTRV2:*LIC-ORF10/20* plants were moved from 20°C to 24°C. After two weeks, it was found that the new grown leaves of the double silenced plants showed normal growth (Fig. 2-11). ‘No.334’1 and ‘*sy-2*’ plants were used as control.

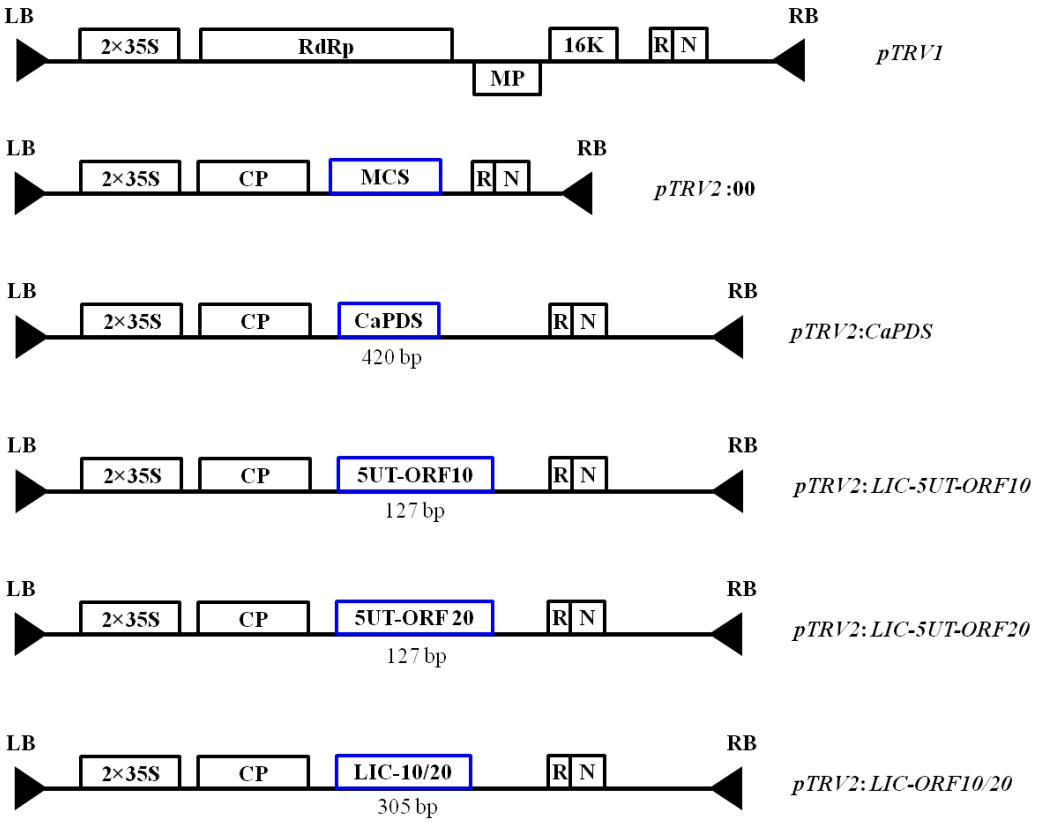

Fig. 2-7. TRV-based VIGS vectors used in pepper (*C. chinense*) plants. A 420 bp fragment of CaPDS cDNA, a 127 bp fragment of *ORF10* cDNA, a 127 bp fragment of *ORF20* cDNA, and a 305 bp fragment of *ORF10* and *ORF20* cDNA were cloned into pCAMBIA 2300 vector by ligation-independent cloning. For the VIGS assay, pTRV1 and pTRV2 and its derivatives were introduced into *A. tumefaciens* strain GV3101 by the freeze-thaw method.

Fig. 2-8. Virus-induced gene silencing of *ORF10* and *ORF20* genes in 'No.3341' (*C. chinense*) plants. Infection of wild-type 'No.3341' pepper plants with recombinant pTRV2:*CaPDS* and pTRV2:*00* as control. Infection of wild-type 'No.3341' pepper plants with recombinant pTRV2:*LIC-5UT-ORF10*, pTRV2:*LIC-5UT-ORF20*, and pTRV2:*LIC-ORF10/20*. Virus-induced gene silencing of endogenous *CaPDS* gene showed photobleaching phenotype.

Table 2-5. VIGS frequency in pepper plants.

Transformant	Silencing plants/total plants	Description
'No.3341'	-	In 'No.3341' plant exhibits normal growth characteristics.
pTRV2:00	-	Plants infected with pTRV2:00 showed normal growth characteristic with slightly shrunken leaves.
pTRV2:*CaPDS*	3/4	Plants infected with pTRV2:*CaPDS* had symptoms of photobleaching consistent with silencing of PDS.
pTRV2:*LIC-5UT-ORF10*	7/9	Plants infected with pTRV2:*LIC-5UR-ORF10* displayed moderate growth retardation of newly emerging leaves. Emerging true leaves showed irregular shape and loss of pigmentation.
pTRV2:*LIC-5UT-ORF20*	4/9	Plants infected with pTRV2:*LIC-5UR-ORF20* displayed moderate growth retardation of newly emerging leaves. Emerging true leaves showed slight loss of pigmentation.
pTRV2: *LIC-ORF10/20*	9/10	Plants infected with pTRV2:*LIC-ORF10/20* displayed severe growth retardation. Emerging true leaves showed loss of pigmentation, narrow, asymmetric and thicker growth, which is similar to the growth of leaves of the '*sy-2*' mutant grown at 20°C.

*The number of plants with the silenced phenotype was determined 21 dpi.

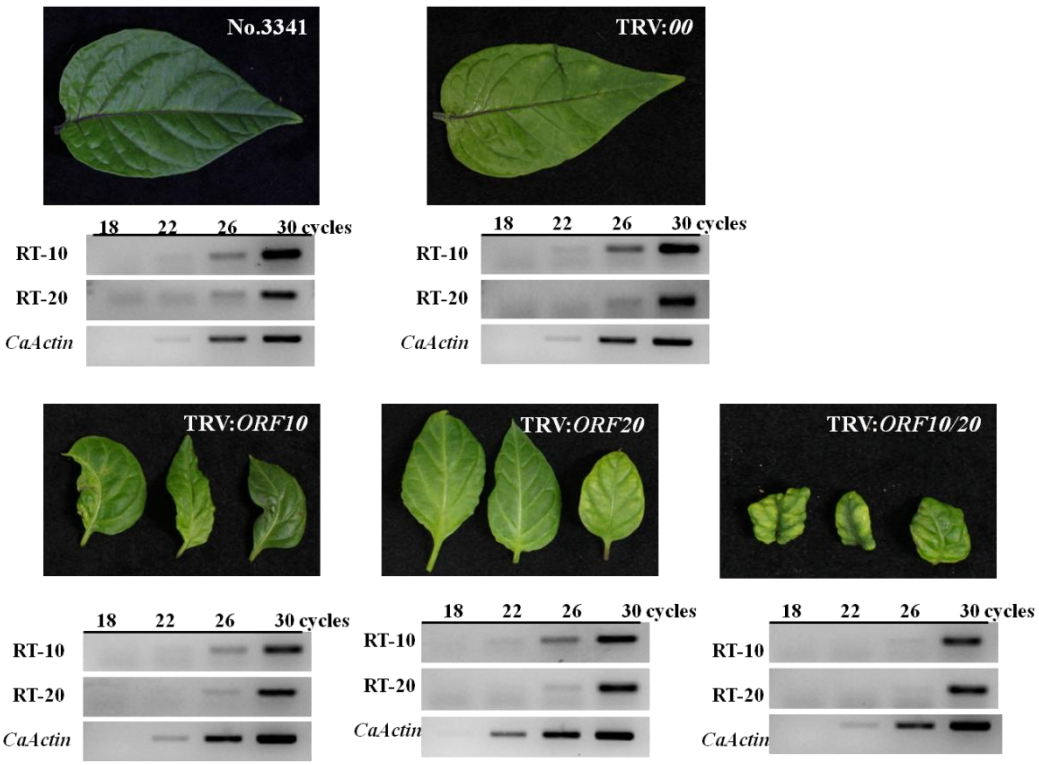

Fig. 2-9. RT-PCR analysis the gene expression of *ORF10* and *ORF20* in silencing plants. *CaActin* was used as a control.

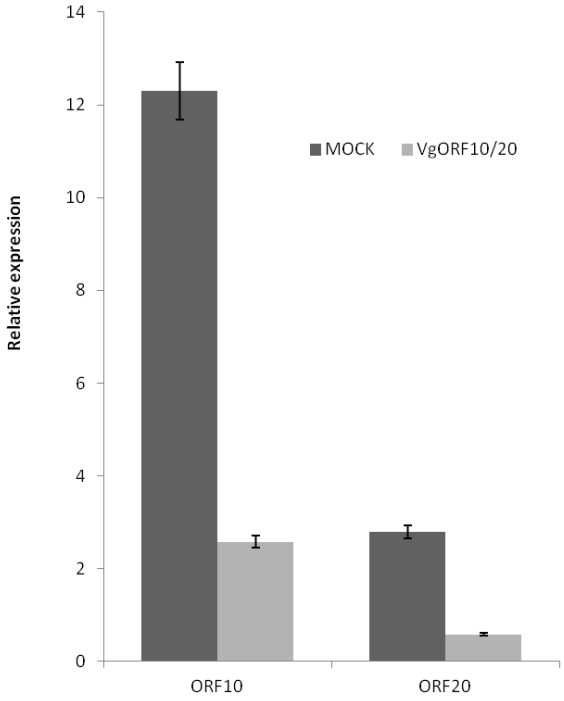

Fig. 2-10. qPCR analysis of double silenced pTRV2:*LIC-ORF10/20* plants. The pTRV2:00 plant (MOCK) was used as control.

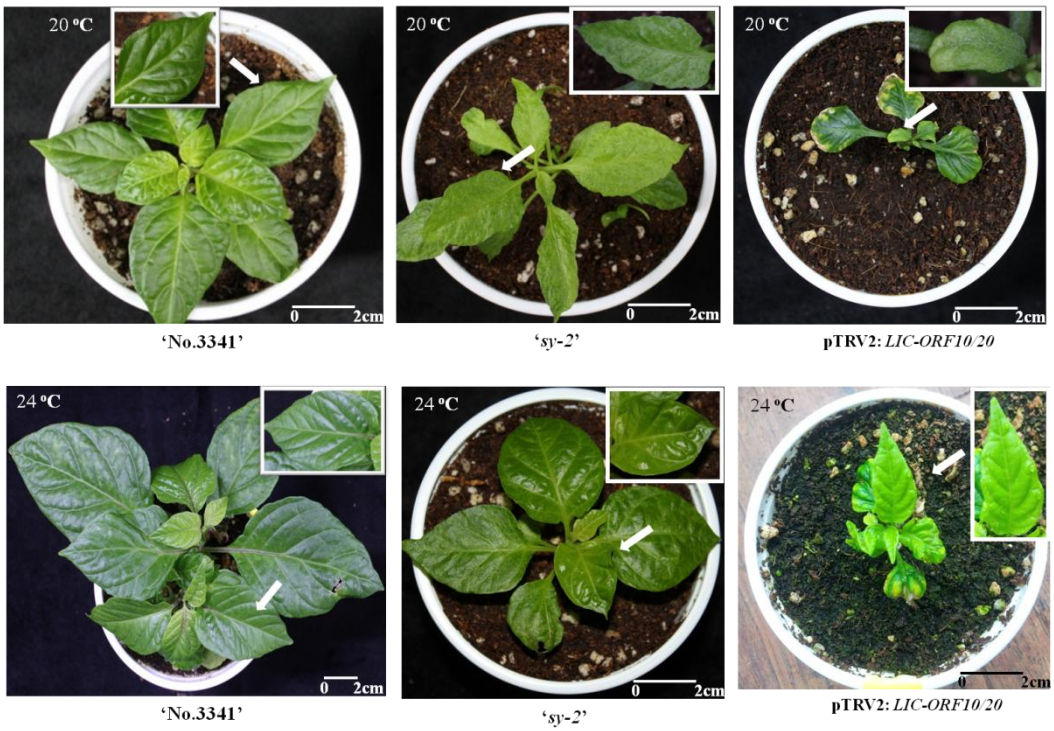

Fig. 2-11. Abnormal growth phenotype in double silenced plants showed recovery at normal growth temperatures of 24°C. The 'No.3341' and '*sy-2*' plants were used as control.

Promoter analysis

Promoters play an essential role in controlling gene expression. It is at this site that the RNA polymerase binds for transcription. Transcription factors play a central role in gene regulation and there are many databases that are dedicated to them. Promoter analysis software works with such databases to predict and identify characteristics of the queried putative promoter sequences.

We used Softberry software running TSSP (Promoter prediction program for plant genes) and NsitePL (Search tool for functional motifs in plant promoter sequences) programs. There is one predicted promoter in *ORF10* and *20*' upstream or at the 5' end of the transcription initiation site. Based on this result, we designed two pair of primers to sequence the promoter region (Table 2-4). The common structure of the promoter is showed in figure 2-12. The promoter region sequencing results were compared between wild-type plants and '*sy-2*' plants. There is one SNP within the promoters of *ORF10* and one 42bp deletion in *ORF20* promoter region (Fig 2-13). The cloning sequences were analysed by Genomatix software suite (Trial Version): MatInspector: Search for transcription factor binding sites (Fig 2-14). In mutant *ORF10*, an SNP at nucleotide 435 has created a MYB-like protein binding consensus sequence, which is not predicted to occur in the WT sequence. Mutant *ORF20* contains a 42 nucleotide deletion at the

proximal end of the promoter which is predicted to eliminate four putative transcription factor binding motifs.

Table 2-6. Primers used for promoter analysis.

Location	Primer name	Primer sequences	PCR size (bp)
	G10-2F	CTGCTGATCATAATCTATCTTCTTCACGAC	1205bp
ORF10	G10-2R	TCATGTCATTGTATATAGGCATAGATTCGG	1205bp
	G20-2F	CTGCTGATCATAATCTATCTTCTTCACGAC	1216bp
ORF20	G20-2R	CTTTGATCGTTGATCTTACCTATGTGGC	1216bp

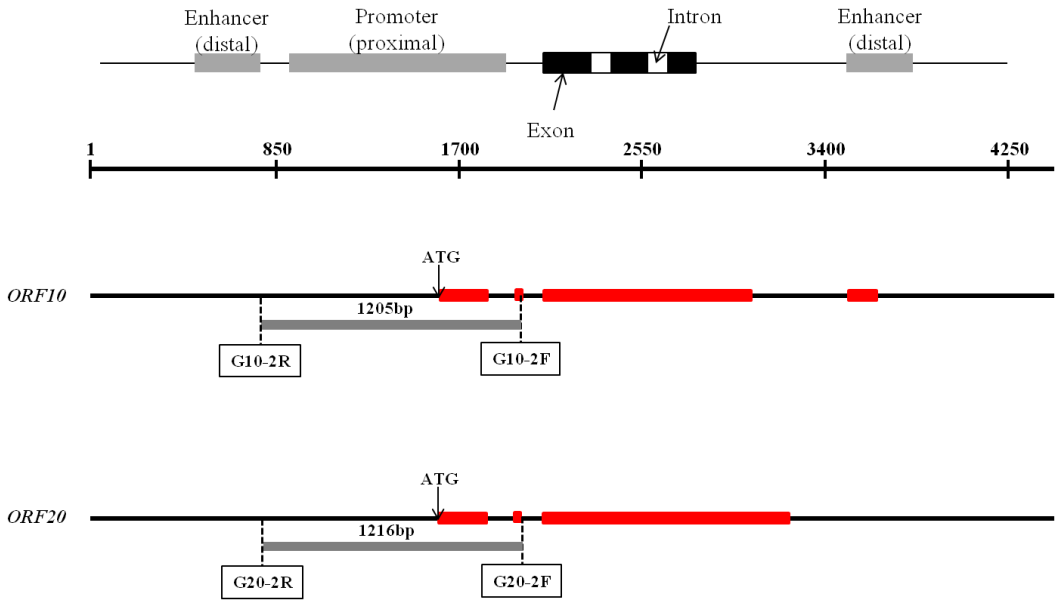

Fig. 2-12. The structure of gene and transcription control regions.

a


gene10WT TCATGTCATTGTATATAGGCATAGATTCGGCTGTGACAGTGTATTTGATATCCAATAAAC
gene10sy TCATGTCATTGTATATAGGCATAGATTCGGCTGTGACAGTGTATTTGATATCCAATAAAC
gene10WT CTTTAGTTACATCTATATTCAACTGTTTATACAATTCAGCAACAAATACACCGTAAGTAA
gene10sy CTTTAGTTACATCTATATTCAACTGTTTATACAATTCAGCAACAAATACACCGTAAGTAA
gene10WT AGCAGAATCGAACAAAACTCCATTGATGATGAAATTCTTAAATTCAACCTCAAATATCCA
gene10sy AGCAGAATCGAACAAAACTCCATTGATGATGAAATTCTTAAATTCAACCTCAAATATCCA
gene10WT TTCACCTCCATATTTAATCATTATTGGTACTAAAACAGAAGTTGTATCCATTTTTTTGTA
gene10sy TTCACCTCCATATTTAATCATTATTGGTACTAAAACAGAAGTTGTATCCATTTTTTTGTA
gene10WT TCAATTGTGAGCAAATTTTTAGAGATTTATACAGGATTGTGGAATGTAAAAGGTAAAGAT
gene10sy TCAATTGTGAGCAAATTTTTAGAGATTTATACAGGATTGTGGAATGTAAAAGGTAAAGAT
gene10WT GAGTATCAGAAAAGGTAATGAACGGAGAATTAGGTGTTTGAAGGGAAAGAAATTTGTGAT
gene10sy GAGTATCAGAAAAGGTAATGAACGGAGAATTAGGTGTTTGAAGGGAAAGAAATTTGTGAT
gene10WT TGAAAAGAAAAGAAGGTAAAGTTGTAATTTGATTTTTTTATTTTTATTTTCCCGTTATAT
gene10sy TGAAAAGAAAAGAAGGTAAAGTTATAATTTGATTTTTTTATTTTTATTTTCCCGTTATAT
gene10WT GCAGTTAATGTGGAAAGGCTATTTAGCGGGTTTATAATGTGACTCGCGAGTGGCTACTTA
gene10sy GCAGTTAATGTGGAAAGGCTATTTAGCGGGTTTATAATGTGACTCGCGAGTGGCTACTTA
gene10WT CGGGTATTACAAATTTGGATGCTAATTTTATAGAATTATTTTATTTTAGGTGTTATTTTT
gene10sy CGGGTATTACAAATTTGGATGCTAATTTTATAGAATTATTTTATTTTAGGTGTTATTTTT
gene10WT GTCCTTTCCCCTAATTTTTTTTTTTTTTTTTTTTTTTTTG-TATAAAGACACAAATTTTGA
gene10sy GTCCTTTCCCCTAATTTTTTTTTTTTTTTTTTTTTTTTTGTATAAAGACACAAATTTTGA
gene10WT AAATGAGGAATGGGTTGGAATTATAGTTTGGGCGTGTGTTTTACTTTACTAAATTGTACA
gene10sy AAATGAGGAATGGGTTGGAATTATAGTTTGGGCGTGTGTTTTACTTTACTAAATTGTACA
gene10WT GTGAGTTTGCAGTTGACCCGACCCATTACGTTTGTCCAATTAGAAAGCGGTTATCCATCC
gene10sy GTGAGTTTGCAGTTGACCCGACCCATTACGTTTGTCCAATTAGAAAGCGGTTATCCATCC
gene10WT ATCCAGCCCAGTTGTGCTAAAATTGCCTAGTCTAAACCCATCTAAAATGAAGAGAGCCAT
gene10sy ATCCAGCCCAGTTGTGCTAAAATTGCCTAGTCTAAACCCATCTAAAATGAAGAGAGCCAT
gene10WT TATTCCGCCGTGTTCATCTGAAGGGTTTTCGGAATCCA
gene10sy TATTCCGCCGTGTTCATCTGAAGGGTTTTCGGAATCCA

Fig. 2-13 Promoter sequence alignment. (a) and (b) are cloning sequence alignment. In *ORF10*, a SNP was found compared to wild-type in '*sy-2*'. In *ORF20*, there is a 42bp deletion compared between wild-type and '*sy-2*'.

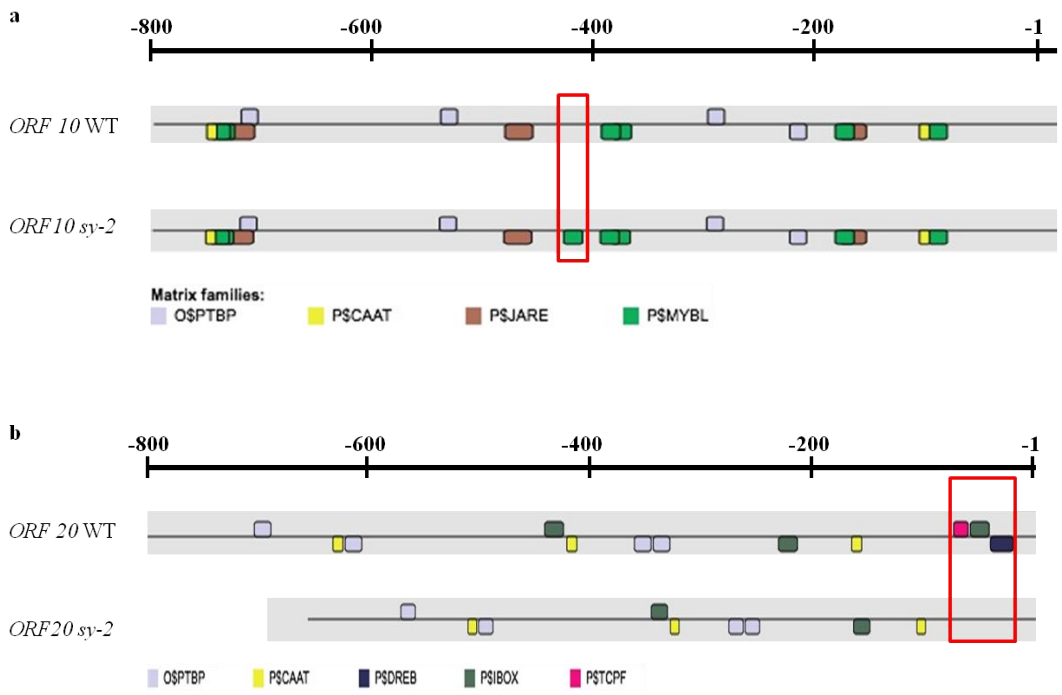

Fig. 2-14 Search for transcription factor binding sites. (a) and (b) are predicted transcription factor binding sites. PTBP- Plant TATA binding protein factor; CAAT- CCAAT binding factors; JARE- Jasmonate response element; MYBL- MYB-like protein domain; DREB - H. vulgare dehydration response factor I - Dehydration responsive element binding factor; IBOX- Plant I-box site; TCPF - TCP class I transcription factor (TCP-Domain).

DISCUSSION

Map-based cloning approaches utilize the relationship between a gene and a marker as the basis for locating a gene. Recently, with the availability of Whole Genome Sequencing (WGS) projects and the development of saturating marker technologies, map-based cloning can now be performed at a higher resolution and the identification of candidate genes in plants is more efficient.

In our previous studies, the results showed that the *sy-2* gene was located in an interval of 138.8 kb between markers SNP5-5 and SNP3-8 on chromosome 1 (see Fig 2-3d, 2-4). Among the *sy-2* candidate genes within this locus, expression levels of two genes predicted to encode F-box proteins and members of the SCF complex, *ORF10* and *ORF20*, were significantly lower in '*sy-2*' plants in both stressed and non-stressed conditions compared to wild-type plants. This finding raised the possibility that these genes are associated with the cold temperature-stress response in the '*sy-2*' pepper.

Virus-induced gene silencing of these ORFs are further support their identity as the *sy-2* gene. At the restrictive temperature, plants silenced for *ORF10* displayed emerging true leaves with an irregular shape and loss of pigmentation. Although not as pronounced, plants silenced for *ORF20* were also displayed moderate growth retardation compared to the control and new true leaves also

showed a slight loss of pigmentation. When both genes were silenced, however, plants showed severe growth retardation and emerging true leaves showed a loss of pigmentation, and exhibited narrow, asymmetric, and thicker growth, which is consistent with the growth of leaves of the '*sy-2*' plant at the restrictive temperature. It was found that the growth phenotype of the double silenced *ORF10/20* plant was reversible, which is consistent with the observation for '*sy-2*' in An et al (2011).

Previously, two F-box genes were mapped to *Ctb1*, a quantitative trait locus (QTL) for cold tolerance in rice (Saito et al., 2004). Later studies showed that cold-sensitive rice plants (Hokkai241 and BT4-74-8) overexpressing an F-box protein with C-terminal kelch repeats from a cold-tolerant variety (Norin-PL8) exhibited cold tolerance (Saito et al., 2010). These results further suggest that the F-box genes *ORF10* and *ORF20* could be candidate genes for *sy-2*.

The SCF complex mediates the ubiquitination of proteins destined for proteasomal degradation, and participate in a wide range of plant developmental processes, such as hormone signaling, circadian rhythms, morphogenesis, embryo development, and senescence (Moon et al., 2004; Schumann et al., 2011; Vierstra, 2003). Mutants of components of the multisubunit E3 ligases show broad pleiotropic effects on plant growth and development (Gray et al., 1999; Liu et al., 2004). For instance, Liu et al. (2004) demonstrated important roles for

Arabidopsis Skp-like genes (ASKs), *ASK1* and *ASK2*, in plant growth and development through analysis of the *ask1 ask2* double mutant, which displayed developmental defects in embryogenesis and seedling growth caused by alterations in cell division, expansion, and elongation. In another study, male-sterility in an *Arabidopsis* mutant with abnormal microspores was found to be caused by an insertional mutation in the *ASK1* gene (Yang et al., 1999). Mutations in *AXR6*, which encodes the SCF subunit CUL1, caused auxin-related defects throughout the plant life cycle (Hellmann et al., 2003). Taking these observations into account, the observed *sy-2* mutant phenotype might be due to defects in the F-box genes and/or the impairment of their interaction with other SCF complex proteins. Furthermore, proteasome-mediated protein degradation plays a crucial role in plant adaptation to environmental changes and maintenance of homeostasis by degrading transcriptional activators or repressor proteins to regulate gene expression (Santner and Estelle, 2010). Our study documented that the altered expression of many genes involved in biological processes, such as cellular process, response to stimulus, and response to stress, is associated with the pepper leaf abnormal phenotype under low temperature. This altered gene expression may be due to changes in proteasome-mediated protein degradation.

ORF10 and *ORF20* were predicted to encode F-box proteins containing C-terminal kelch repeats with a high degree of conservation with other F-box/kelch

proteins from plants (Fig. 2-2). Two amino acid changes were observed within the kelch repeats containing domain of the *ORF10* gene from '*sy-2*' mutant plants, which could affect protein-protein interactions (Jain, 2007; Schumann et al., 2011; Xu et al., 2009). Ubiquitination is a post-translational modification process that mediates many aspects of plant growth and development (Lee and Kim, 2011; Lyzenga and Stone, 2012). Typically, substrate recognition by ubiquitin ligases requires an initial substrate modification through phosphorylation; F-box proteins recruit phosphorylated substrates to the SCF complex for ubiquitination (Skowyra et al., 1997). However, SCF can also be regulated through phosphorylation of the F-box protein itself (Kato et al., 2010; Santra et al., 2009). The mutation in the *ORF20*-encoded protein causes an amino acid change from S to K, raising the possibility that it affects the phosphorylation status of the protein, its subsequent binding of the substrate to the SCF complex, and its ultimate degradation via the proteasome (Kato et al., 2010). Identification and validation of protein-protein interaction and regulatory sites are essential for understanding their functional consequences. Based on previous observations and those presented here, we suggest that the F-box genes *ORF10* and *ORF20* are the most likely candidates for the cold-sensitive *sy-2* gene and are associated with abiotic stress responses in pepper. Further functional analysis of the *ORF10* and *ORF20* genes will help to

unravel the molecular mechanism responsible for the *sy-2* cold temperature growth phenotype.

Regulation of gene expression as a consequence of external stimuli is mediated at the transcriptional, post-transcriptional, and post-translational level. However, transcriptional regulation plays the greatest role in the activation and suppression of expression, and is largely controlled through gene promoters and their contributing cis-acting elements (Hernandez-Garcia and Finer, 2014). Typically, a plant promoter can be divided into proximal and distal regions, whereby the proximal region contains the conserved TATA box approximately 25 to 30bp downstream of the transcriptional start site. This region is the binding site for the transcription initiation factor TFIID TBP (TATA-box-Binding Protein) subunit. The distal region can contain multiple cis-acting regulatory response elements that contribute to the complex expression profile of a particular gene. These elements have been shown to play an important role in a plants response to abiotic stress. Common stress-responsive elements comprise the dehydration-responsive element DRE (A/GCCGAC) implicated in the regulation of cold and dehydration responses in Arabidopsis, the low temperature responsive element C-repeat binding factor (CBF), and the ABA responsive element ABRE (ACGTGG/T) that regulates dehydration and salinity responses in Arabidopsis and rice (Hernandez-Garcia and Finer, 2014). Several transcription factor genes

i.e. *MYB*, *CBF/DREB1*, *HSF*, *TGA6*, *BOS1*, *bZIP*, *AP2/EREBP* etc. are indicated for their role in plant development and stress tolerance (Ambawat et al., 2013).

Our data suggests the presence of a number of cis-acting response elements in the promoters of *ORF10* and *20*. In *ORF10*, there are several putative Jasmonate response elements present, which is of significance because Jasmonate has been widely reported as a mediator of abiotic stress in plants (Sharma et al. 2016). Moreover, in mutant *ORF10*, an SNP at nucleotide 435 has created a MYB-like protein binding consensus sequence, which is not predicted to occur in the WT sequence. MYB proteins are known to be involved in plant development, abiotic stress tolerance, hormone signaling and disease resistance (Peng et al., 2016). Mutant *ORF20* contains a 42 nucleotide deletion at the proximal end of the promoter which is predicted to eliminate four putative transcription factor binding motifs. Of particular interest is the elimination of a Dehydration Response Element. The APETALA2/Ethylene-Responsive Factor (AP2/ERF) superfamily of transcription factors have been shown to regulate diverse plant responses. The ERF family is further subdivided into the ERF and the C-repeat-binding factor/dehydration responsive element binding proteins (CBF/DREB). In dicotyledonous plants, CBF/DREB proteins bind to the C-repeat/dehydration-responsive-element (CRT/DRE) cis-acting element in the promoters of target genes and are usually associated with responses to abiotic stress and the

phytohormone abscisic acid (Dey and Corina Vlot, 2015). The presence of this response element in the promoter of *ORF20* suggests the gene may be regulated in response to abiotic stress through dehydration responsive element binding proteins. However, with respect both ORF10 and ORF20 a more detailed study of the promoter region will need to be conducted to ascertain with any degree of certainty if low temperature cis-acting response elements are responsible for the regulation of these genes.

In summary, in the present study, a genetic and physical map of the temperature-sensitive gene *sy-2* was constructed to identify the candidate gene for temperature sensitivity. Two putative F-box genes found in the *sy-2* region were considered strong candidates for the *sy-2* locus. The '*sy-2*' plants exhibited abnormal phenotypes when exposed to low temperature (20°C) and showed lower expression levels of these F-box genes than wild-type plants, even at 28°C, suggesting their function in growth and development under low temperature conditions. Recently, there has been remarkable progress in understanding the ubiquitin-proteasome system and their roles in cellular processes. Biochemical and molecular studies have shown that these proteins can form complexes or supercomplexes to regulate the degradation of different cellular proteins. Therefore, we hypothesize that the *sy-2* candidate F-box proteins and their interacting protein partners serve an important role in plant growth and

development under low temperature conditions. Further functional characterization of these F-box genes is warranted to resolve the molecular and physiological mechanisms of temperature sensitivity in pepper. Furthermore, the findings and resources generated herein will facilitate marker-assisted selection for cold tolerance in pepper.

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CHAPTER III

Mitochondrial-targeted Expression of *orf456* Causes Male Sterility in Chinese Cabbage (*Brassica rapa*)

ABSTRACT

Cytoplasmic male sterility (CMS) is a phenomenon specific to plant reproduction that has been extensively exploited for hybrid seed production. *Orf456* was previously identified as a candidate gene mediating male sterility in pepper. To gain further insight of this candidate gene, we carried out experiments to transform Chinese cabbage (*Brassica rapa* L.). About 30 T_1 transgenic lines were obtained and approximately 50% of T_1 transgenic Chinese cabbage lines showed male sterility. To evaluate pollen viability, three approaches including plasmolysis test, staining pollen, and *in vitro* germination assay were used. Analysis of the CMS transgenic lines showed that transgenic Chinese cabbages produced aberrant pollen development while some were unable to produce pollen. In conclusion, the mitochondrial *orf456* gene could induce partial male sterility in transgenic Chinese cabbage. Nevertheless, how the *orf456* gene precisely functions to induce male sterility and its biochemical function remains to be discovered.

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited condition in which a plant is unable to produce viable pollen (Gómez, 1999). Since the first documentation of male sterility by Joseph Gottlieb Kölreuter, who observed anther abortion within species and specific hybrids, CMS has been identified in over 150 plant species (Schnabel and Wise, 1998; Hanson and Bentolila, 2004; Chowdhury et al., 2010).

CMS is caused by the extranuclear genome (mitochondria or chloroplast) and shows maternal inheritance. Manifestation of male sterility in CMS may be controlled either entirely by cytoplasmic factors or by interaction between cytoplasmic and nuclear factors. CMS can occur at different stages during reproductive developments (Linke and Börner, 2005). CMS is generally considered to be caused by the rearrangement of mitochondrial genomes, which, in many cases, is attributed to the generation of novel open reading frame (ORF) (Yang and Zhang, 2007). In a number of cases, transcripts originating from these altered ORFs are translated into unique proteins that appear to interfere with mitochondrial function and pollen development (Yamamoto et al., 2008). Anther degeneration in CMS is probably caused by dysfunction of tapetum or vacuolation of tapetum, pollen mother cells (PMC), and microspores (Shi et al., 2010). Most

studies have been performed on vegetative tissues that express the CMS-associated proteins. However, biochemical and physiological studies have provided some clues to the disruption in aborted pollen development (Shi et al. 2010). Nevertheless, the precise action of a mitochondrial locus that confers male sterility has not been determined definitively for any species.

The Brassicaceae family contains about 3,500 species and 350 genera and is one of the economically most important plant families (Quijada et al., 2007). *Brassica rapa* (2n =20) is a diploid crop in this family, which is currently widely cultivated in China, Japan, Korea, India, Canada, and Europe to produce vegetable, oilseed, and fodder (Hirai and Matsumoto, 2007; Li et al., 2013). Since Ogura (1968) first reported the CMS line of radish, a number of F_1 hybrid cultivars have been developed using the Ogura CMS system in many economically important Brassica family crops. Despite many studies on CMS, researchers and breeders have made efforts to search for new sources of CMS in breeding.

In the previous studies, a mitochondrial *atp6* gene was identified as a CMS associated gene (Kim and Kim, 2006; Kim et al., 2001). Then a second CMS-associated gene *orf456*, which located at the 3'-end of the *CoxII* gene in CMS pepper (Kim et al. 2007). Later, overexpression of *Orf456* was shown to severely induce male sterility in *Arabidopsis* (Kim et al., 2007). To investigate the

functional role of the ORF456 protein in *B. rapa*, we transformed *B. rapa* with *orf456* fused to a mitochondrion-targeted *CoxIV* gene.

MATERIALS AND METHODS

Plant materials

The wild type ‘Seoul Baechu’ (*B. rapa*), were kindly provided by D.H. Kim at Seoul National University, Korea.

Plant chimeric gene construction and transformation

Orf456 and *CoxII* were cloned into the plant transformation vector pMDC32. Purified plasmid DNA of each pMDC32 construct (Fig. 3-1a) was electroporated into *Agrobacterium. tumefaciens* LBA4404. The presence and stability of the transgenes was verified by PCR (Fig. 3-1a). Chinese cabbage cultivar ‘Seoul’ was transformed using leaf disk method at National Institute of Horticultural and Herbal Science, Wanju, Korea. For the T_1 selection, hygromycin (15mg/L) containing 50% Murashige and Skoog (MS) medium (Sigma, St.Louis, Mo, USA) was used.

Growth conditions and sampling

Seeds of transgenic Chinese cabbage lines were sterilized with 70% ethanol and 50% Clorox and germinated on MS media (Sigma) containing Hygromycin B (HTP) (Sigma). After 2 weeks, the seedlings were transplanted to soil and grown

in a growth chamber for 2 or 3 weeks. When the fifth true leaf was fully expanded, the seedlings were exposed to 4°C in a period of 20 to 30 days to achieve a complete vernalization for bolting (National Institute of Horticultural and Herbal Science).

Fresh buds and anthers were collected and fixed in Carnoy's fluid (a fixative composed of 60% ethanol, 30% chloroform, and 10% glacial acetic acid) and samples were stored in a refrigerator at 4℃. Fresh pollen of the *orf456* transformants and wild-type line were collected by shaking a flower on a clean sheet of paper, pouring the pollen into a 1.5 mL microcentrifuge tube, added the pollen with 0.5 mL of 70% (v/v) ethanol and stored at 4°C.

DNA and RNA extraction, and RT-PCR analysis

Genomic DNA was extracted from young leaves using CTAB method (Prince et al. 1997). Total RNA was extracted from young flowers buds of T_1 generation transgenic plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was then treated with RNase-free DNase (Ambion,Austin, Texas, USA) to remove possible contamination of genomic DNA for RT-PCR. Total RNA (2 μg) was used for first-strand cDNA synthesis using M-MLV reverse transcriptase (Invitrogen). For PCR analysis of *orf456*, the forward primer 5'-ATGCCCAAAAGTCCCATGTA-

3’ and the reverse primer 5’-TTACTCGGTTGCTCCATTGTTT-3’ were used. For *CoxIV*, the forward primer 5’-GGATCCATGTTGTCACTACGTCAAT CTATAAGA-3’ and the reverse primer 5’-CCCGGGACCCTCTTTAGCACCA GGACC-3’ were used. For *HPT*, the forward primer 5’-CCTGAACTCA CCGCGACG-3’ and the reverse primer 5’-AAGACCAATGCGGAGCATAT-3’ were used. *Brassica* actin primers were used as control.

Pollen viability test

Plasmolysis test was performed to evaluate pollen viability. Pollens of *orf456* transformant lines and wild-type plants were placed in hypertonic (25% glycerol) solution to test whether the pollen grains are alive. Fresh pollen of the *orf456* transformant and wild-type lines were collected and added 20 μL of hypertonic solution (20% glycerol) on glass slides. After 10 min incubation at room temperature, pollens were observed under a light microscope. Pollen were stained with 1% aceto-carmine (Nassar et al., 2000) and iodine (Yu et al., 2010) to detect cytoplasmic content. After 10 min incubation at room temperature, stained pollens were observed under a light microscope. *In vitro* germination assays were performed to determine the germination ability of pollen. Growth medium contained 10% sucrose, and 0.01% boric acid (pH 6.8). After incubation at 20-

25°C for about 4 hrs, the solution has been covered with a glass cover slip and photographed.

RESULTS

Chimeric gene construction and transformation

In the previous study, a CMS-associated gene, *orf456* was found at the 3'-end of the *CoxII* gene, which was identified as a strong candidate for determining the male-sterile phenotype in pepper (Kim et al., 2007). When this gene was ectopically expressed in *Arabidopsis*, transgenic plants showed abnormal pollen development and sterility. To test transgenic expression of the *orf456* gene can also induce male sterility in *Brassica*, transgenic plants were developed. To transform *B. rapa*, a chimeric gene was constructed by fusing *orf456* gene with a mitochondrial transit peptide sequence (54 codons) of the nuclear *CoxIV* gene. The *orf456* gene was amplified from CMS lines of chili pepper and ligated after the mitochondrial-targeting sequence of *CoxIV*. Subsequently, the chimeric gene has been ligated to the plant transformation vector, pMDC32, driven by the 35S promoter. The schematic structure of the chimeric *orf456* gene is shown in Fig. 3-1a. The constructs were introduced into *B. rapa* 'Seoul Baechu'. Putative transgenic plants were selected by PCR and RT-PCR and ten T_0 plants exhibiting male sterility were obtained (Fig. 3-2). We used the *orf456* gene as a marker to proof that this gene was inherited and could be expressed in T_1 transgenic (Fig. 3-2). The male-sterile phenotype was shown to be genetically transmitted to the T_0

and T_1 generation associated with *orf456* expression in *B. rapa* (Fig 3-2). All the ten male-sterile T_1 plants expressed the *orf456* gene, and all the 30 male-fertile T_1 plants lacked *orf456* expression in *B. rapa*.

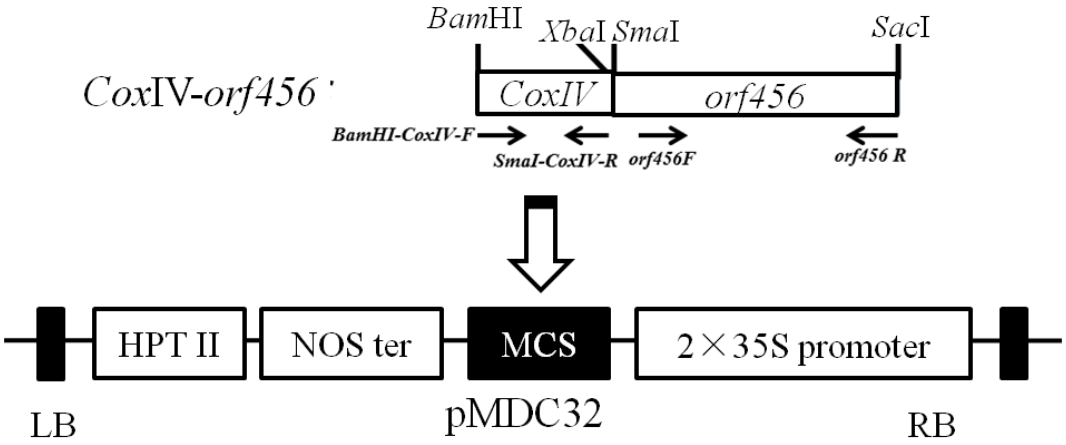

Fig. 3-1. *Orf456* chimeric gene construct and transformation in *B. rapa*. Schematic diagram illustrating the construction of *orf456* and *CoxIV* were ligated to the plant transformation vector, pMC32, driven by the 35S promoter.

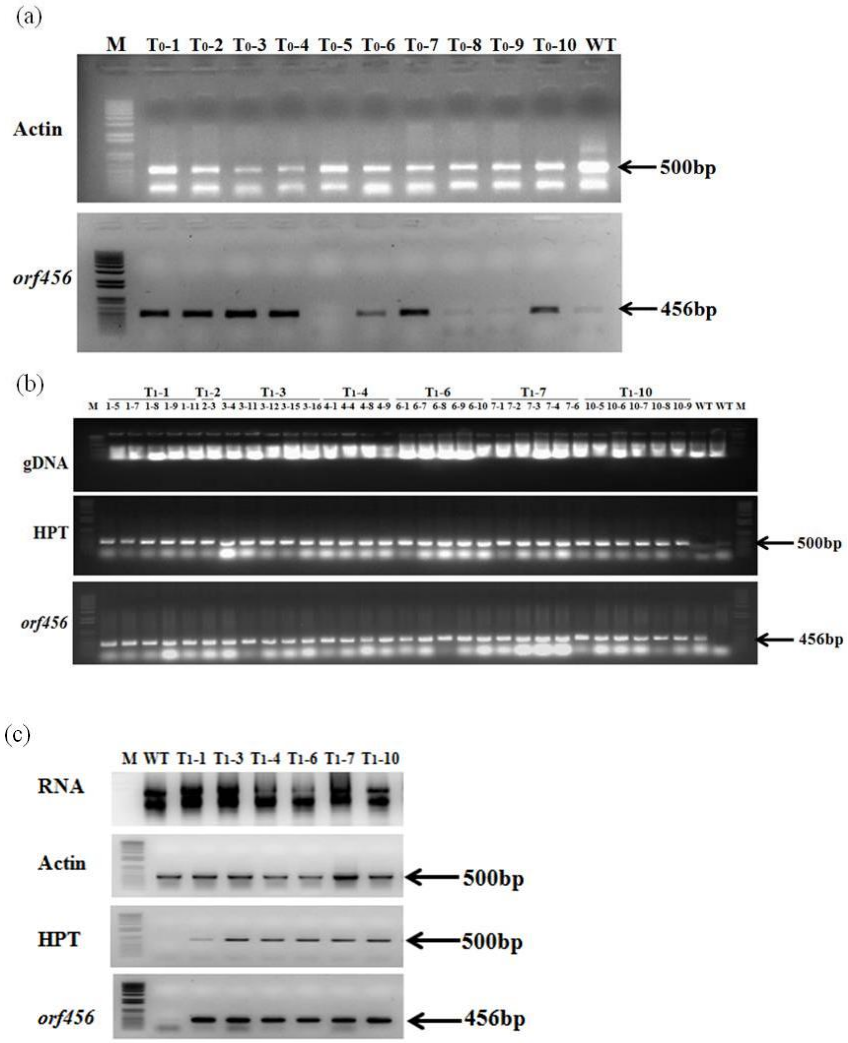

Fig. 3-2. PCR and RT-PCR results in transgenic lines. (a) Polymerase chain reaction (PCR) analysis of chimeric gene genetically modified situation in T_0 individual transgenic *B. rapa* plants. Actin was used as internal control to probe Chinese cabbage cDNA quality. (b) PCR analysis of chimeric gene in T_1 individual transgenic *B. rapa* plants. HPT was hygromycin B, an antibiotic produced by the bacterium *Streptomyces hygroscopicus*. (c) RNA and RT-PCR analysis of T_1 individual transgenic *B. rapa* lines.

Phenotypes of *orf456* transformants

Morphological characteristics were observed in transgenic plants harboring the chimeric *orf456* gene. Vegetative growth of transgenic *B. rapa* was similar to that of wild-type plants, but their reproductive characteristics differed in numerous ways including delays in bolting and flowering time (Fig. 3-3a), aborted buds structures at the basal part of the raceme (Fig. 3-3b), and aberrant floral organ (Fig. 3-3c). Transgenic *B. rapa* showed a lack of seed-set in at early developmental stage (Fig. 3-3d, e). Seed size and weight also differed between wild-type and transgenic *B. rapa*. Wild-type seeds were spherical, brown, and glossy, which the transgenic ones showed shriveled shape seeds, implying embryo hypoplasia (Fig. 3-3f). When the transgenic plants were cross-pollinated with wild-type plants, seed-set occurred indicating normal pistil fertility (Fig. 3-3e).

Additionally, four types of aberrant flowers were observed in transgenic plants; flowers with less or more than six normal stamens (Fig. 3-3c1), flowers with degenerative stamens devoid of pollen (Fig. 3-3c2), one flower with several styles and petal-like sepal (Fig. 3-3c3), and two or more coalescing flowers (Fig. 3-3c4, c5). These phenotypes of aberrant floral organs in transgenic *B. rapa* are similar with others studies (Kim et al., 2007; Yang et al., 2010).

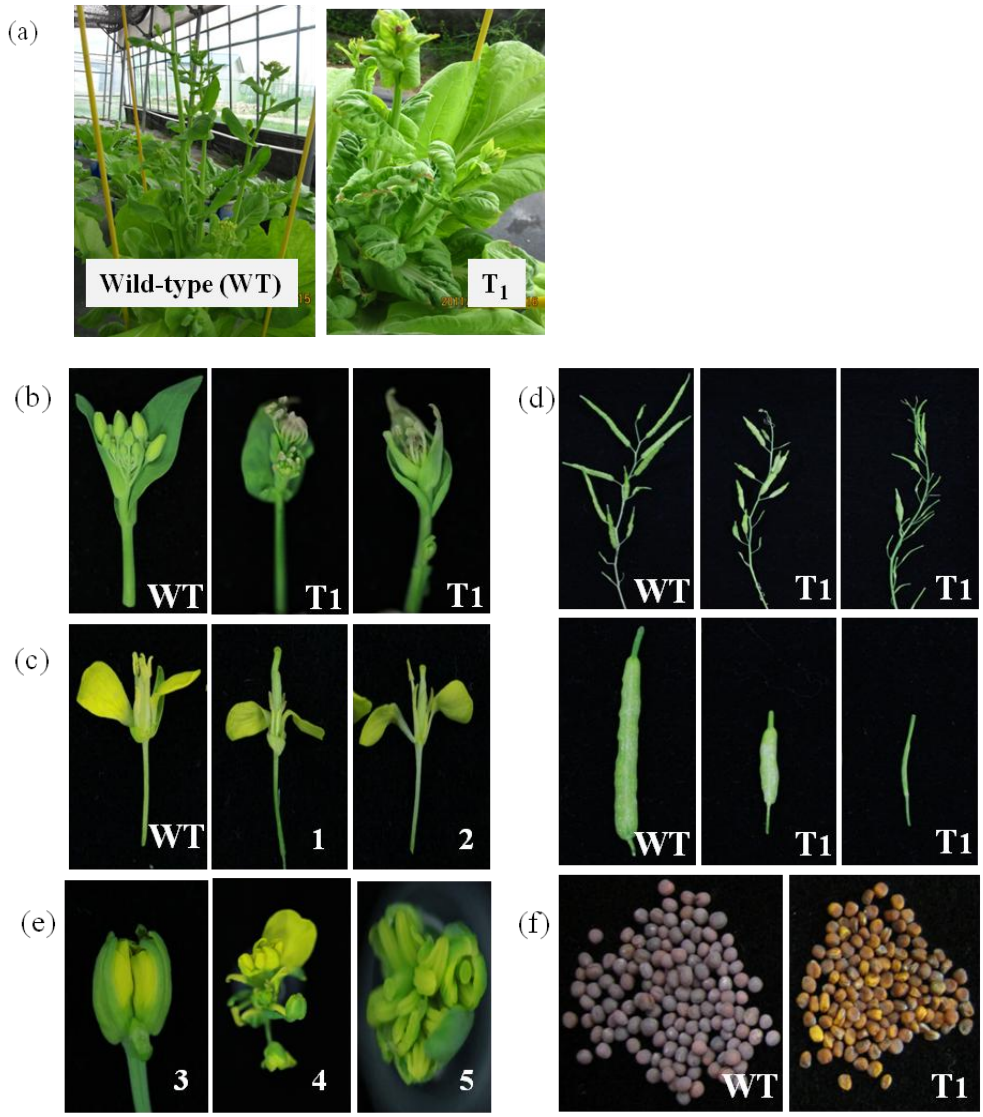

Fig. 3-3. Phenotypes of wild-type and *orf456* transformants. Morphological characters were observed in transgenic plants in which the chimeric *orf456* gene was expressed. In transgenic experiments, the results suggested that the *orf456* may have expression in the evolution of reproductive growth in transgenic '*B. rapa*' lines. (a) Whole plant of transgenic T_1 *B. rapa*, (b) buds, (c) blossoms, (d) siliques, (e) bolting, (f) seeds.

Pollen viability of transgenic lines

We used three different approaches to estimate pollen viability in transgenic plants and wild-type plants: plasmolysis test, staining pollen with dyes and *in vitro* germination assay. The plasmolysis test was performed to check the intactness of pollen. When placed in hypertonic (25% glycerol) solution, pollen of *orf456* transformed *B. rapa* burst within 10-15 min (Fig. 3-3b), while the wild-type pollen grains remained intact even after 24 h (Fig. 3-3a). These results indicated that the pollen of the *orf456* transformed *B. rapa* were ateliosis. Pollen staining was used to determine enzymatic activity and membrane integrity of pollens. The morphology of acetocarmine and iodine stained pollen was altered in transgenic *B. rapa* (Fig. 3-3b). These results indicated that pollen activity of transgenic plants was markedly different (Fig. 3-3b). In vitro germination assays determine the actual germination ability of pollen. After 4 h incubation at 20-25℃, the pollen grain was slightly swollen, with one of the three pores (furrows) open and the pollen tube emerging. The pollens from wild-type *B. rapa* was able to produce pollen tubes, whereas the transgenic plant pollen was observed to burst but no pollen tube was produced (Fig. 3-3c).

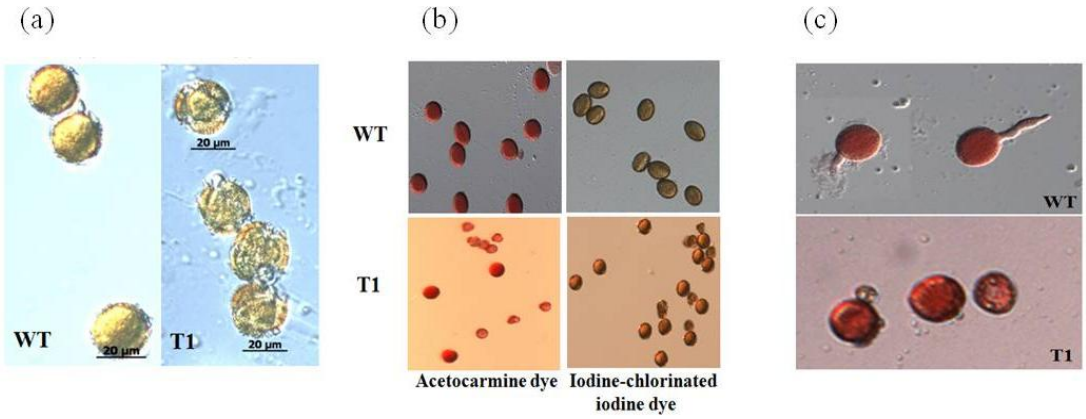

Fig. 3-4. Pollen viability test results. (a) Plasmolysis test was performed to probe the pollen. In hypertonic (25% glycerol) solution, pollen from *orf456* transformants *B. rapa* burst within 10-15 min, while the wild-type pollen grains remained intact even after 24 h. (b) Staining test to determine pollen enzymatic activity and membrane integrity. (c) *In vitro* germination assays determine the actual germination ability of pollen under suitable conditions. After 4 h, *in vitro* germination, pollen from wild type *B. rapa* were able to produce pollen tubes. However, pollen from transgenic *B. rapa* failed to germinate.

Statistical analysis of pollen abortion phenotypes in the CMS lines

To confirm the function of *orf456* in producing a male-sterile phenotype, two independent tests were conducted. We randomly selected seven out of ten transgenic T_0 lines that contained *CoxIV-orf456,* and obtained T_1 transgenic plants. The malformed pollen ratio of 15 plants lower than 10%, which showed a male fertility (Table 3-1). We analyzed the ratio of aberrant flowers and malformed pollen in T_1 *B. rapa* lines. The result indicated that approximately 50% T_1 *B. rapa* were male-sterile phenotype (Table 3-1). Three T_0 generation lines 1, 6 and 10 showed poor seed set ability when compared to the wild-type (Table 3-1). Normal blossoms were compared with aberrant blossoms and we found the aberrant blossoms had a higher malformed pollen ratio. Four T_1 lines 1, 3, 6, and 10 showed higher malformed pollen radio compared with other lines (Table 3-1). Analysis result of seeds and siliques of wild type and transgenic T_1 *B. rapa* plants. We found four T_1 lines 1, 6, 7, and 10 had lower number of siliques and seeds compared with other lines (Table 3-1).

Table 3-1. Analysis of malformed pollens ratio of T_1 *B. rapa* plants.

Brassica rapa	Blossom			Pollen					
				Normal blossom			Malformed blossom		
	Normal	Malformed	Malformed blossom ratio (%)	Normal	Malformed	Malformed pollen ratio (%)	Normal	Malformed	Malformed pollen ratio (%)
WT	25	0	—	824	8	0.96	915	7	0.76
1-5	17	6	26.09	424	49	10.36	417	711	63.03
1-7	22	4	15.38	589	72	10.89	885	199	18.36
1-8	32	1	3.03	394	46	10.45	—	—	—
1-9	22	7	24.14	533	218	29.03	682	243	26.27
1-11	25	0	—	325	187	36.52	—	—	—
2-3	25	0	—	814	62	7.08	—	—	—
3-4	20	4	16.67	731	213	22.56	79	725	90.17
3-11	31	4	11.42	230	349	60.28	6	1291	99.54
3-12	6	1	14.28	229	152	39.89	—	—	—
3-15	12	4	25	574	78	11.96	328	517	61.18
3-16	79	4	4.82	417	57	12.03	5	500	99
4-1	25	0	—	661	46	6.51	—	—	—
4-4	25	0	—	703	57	7.5	—	—	—
4-8	25	0	—	718	49	6.39	—	—	—
4-9	25	0	—	897	42	4.47	—	—	—
6-1	25	0	—	719	34	4.52	—	—	—
6-7	25	0	—	974	29	2.89	—	—	—
6-8	25	0	—	485	40	7.61	—	—	—
6-9	12	9	42.86	636	212	25	355	335	48.55
6-10	25	0	—	712	12	1.66	—	—	—
7-1	12	1	7.69	921	55	5.64	—	—	—
7-2	13	1	7.14	894	210	19.02	—	—	—
7-3	25	0	—	702	27	3.7	—	—	—
7-4	47	13	21.67	528	265	33.42	246	87	26.13
7-6	25	0	—	582	192	24.81	—	—	—
10-5	25	0	—	592	45	7.06	—	—	—
10-6	25	0	—	482	72	12.99	—	—	—
10-7	25	0	—	633	48	7.05	—	—	—
10-8	9	3	25	732	108	12.86	312	270	46.39
10-9	23	1	4.17	505	23	4.36	—	—	—

DISCUSSION

Chimeric *orf* genes resulting from the rearrangement of a mitochondrial genome are generally thought to be a causal factor in the occurrence of CMS (He et al., 1996). Several transformants containing *orf* gene constructs, with or without a mitochondrial targeting sequence, exhibited a semi-sterile or male sterile phenotype (He et al., 1996; Kim et al., 2007; Wang et al., 2006; Yamamoto et al., 2008). The failure of mitochondrial expression of novel *orfs* to induce male sterility is probably due to problems in disruption of mitochondrial membranes (He et al., 1996; Rhoads et al., 1995), effect on ATP synthase function (Bergman et al., 2000; Sabar et al., 2003) and pollen abortion by programmed cell death in transgenic plants. However, little information is available about how the mitochondrial factor regulates the expression of the nuclear genes involved in male sterility. Previously, to provide definitive evidence that expression of an abnormal mitochondrial gene can interrupt pollen development, a CMS associated mitochondrial DNA sequence from chili pepper, *orf456*, was identified by comparison of cytoplasmic genomes in fertile and CMS lines (Jo et al., 2009; Kim et al., 2007). In this paper, *orf456* gene was introduced into Chinese cabbage to test the candidate CMS associated gene function.

Several attempts have been made to induce CMS by intergenic hybrid or transformation of CMS-associated genes into *Brassica*. Ogura (1968) first reported the CMS line of radish, a number of F_1 hybrid cultivars have been developed using the Ogura CMS system in many economically important crops. The *atpA* and *orf220*, newly isolated from CMS stem mustard (*Brassica juncea* var. *tumida* Tsen et Lee) (Yang et al., 2010). A chimeric gene *orf220-atp2-1* was transformed into in wild type stem mustard. Kim et al. (2007) showed that transgenic *Arabidopsis* plants carrying a chimeric *orf456* could induce male sterility phenotype which was similar to those observed in CMS chili pepper. In this study we also showed that chimeric *orf456* gene induce partial male sterility in *B. rapa.*

Disruption in pollen development is thought to be a consequence of mitochondrial dysfunction (He et al., 1996). Furthermore, many genes related to the mitochondrial respiratory complex and pollen development were observed to be down-regulated in transgenic plants and the CMS lines (Yang et al., 2010). To date, functional genes specifically related to pollen development have been well documented. Mutation of any of these genes causes failure of micro-sporogenesis or abortion of pollen (He et al., 1996; Kim et al., 2007; Shi et al., 2010; Yang et al., 2010). We used three different approaches to estimate pollen viability: plasmolysis test, staining pollen with dyes and *in vitro* germination assay

(Tuinstra and Wedel, 2000). The results of our study indicate that introduction of each of the *orf456* gene constructs resulted in aberrant pollen development and disturbance in function (Figs. 3-2, 3-3). We used normal blossoms to compare with the aberrant blossoms and we found the aberrant blossoms had higher malformed pollen ratio.

In conclusion, the mitochondrial *orf456* gene may represent a strong candidate gene involved in CMS in transgenic Chinese cabbage. Nevertheless, how the *orf456* gene functions precisely to induce male sterility and its biochemical function remains to be discovered. In addition, further research is required to explore the signaling pathway of mitochondrial-nuclear genome interactions.

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요약

온도는 작물의 생산성과 지리적 분포에 영향을 미치는 가장 중요한 환경적 요인 중 하나이다. 열대지역 및 아열대 지역에서 유래한 원예 작물들은 온도에 매우 민감하며, 특히 저온 장해를 입을 가능성이 매우 높다. 1989 년 일본 연구진들에 의해 아프리카의 세이첼 섬에서 자생하며, 24°C 이하의 온도에서 기형을 보이는 고추 계통 '*sy-2*' (*C. chinense*, *Seychelles-2*)를 발견되었다. 온도 민감성 유전자 *sy-2*는 이전 연구에서 토마토 염색체 1 번에 위치한 Ch1_scaffold 00106 의 300 kb 지역에 매핑되었다. 본 연구에서는 *sy-2* 유전자를 밝히기 위하여 *sy-2* 유전자좌에 대한 미세유전자 지도 작성 및 염색체지도 기반 클로닝법을 활용하였다.

1 장에서는 두 개의 F2 분리집단을 활용하여 *sy-2* 유전자좌에 대한 미세유전자 지도를 작성하였다. 토마토 스캐폴드(C01HBa0051C14)와

고추 스캐폴드(2607(377.77 kb), 3515(200.85 kb), 2510(318.608 kb) 간의 비교를 통하여 14 개의 SNP 마커와 4 개의 KASP 마커를 개발하였으며, 그 중 SNP5-5 마커와 SNP3-8 마커 사이에 *sy-2* 유전자가 위치하였다. 이 지역은 고추 염색체 1 번의 말단으로, 138.8 kb 의 물리적 거리 이내에 27 개의 유전자가 예측되었다.

2 장에서는 예측한 유전자에 대한 RNA-seq, RT-PCR, qPCR 분석을 진행하였다. RNA-seq 을 이용하여 저온 조건이 주어졌을 때 야생형과 '*sy-2* 계통 간에 발현 차이를 보이는 유전자의 히트맵을 작성하였다. RNA-seq 과 RT-PCR 결과를 통해 '*sy-2* 계통에서 발현이 감소하는 *ORF10* 과 *ORF20* 을 최종 후보 유전자로 선발하였다. qPCR 분석으로 두 유전자의 발현을 확인하였으며, *ORF10* 유전자는 20°C 에서 'sy-2' 계통에 비해 야생형 계통에서 8.2 배 높게 발현하였다. 염기서열 분석으로 *sy-2* 유전자 특이적인 변이 서열을 확인하였으며, 이는

ORF10 유전자의 C231G, N315D 및 *ORF20* 유전자의 S256K 아미노산 서열 변화를 유도하였다. 종합하면 *ORF10* 과 *ORF20* 유전자는 kelch type F-box 단백질을 암호화 하는 것으로 예상되며, 야생형과 '*sy-2*' 계통에서 발현양의 차이를 보였다. VIGS 실험을 진행하여 *ORF10*, *ORF20* 유전자의 발현을 각각 감소시킨 식물체에서는 'sy-1' 계통과 같이 신엽의 생장이 억제되는 현상을 관찰할 수 있었다. 두 유전자를 동시에 억제시킨 식물체에서는 3 주 후 생장이 심하게 억제되었으며, 본엽이 하얗고 길게 자랐으며, 비대칭적이며 두껍게 자라 20°C 에서 자라난 '*sy-2* 계통과 유사한 외형을 보였다.

3 장에서는 고추의 세포질적 웅성불임을 유도하는 유전자인 orf456 의 기능을 알아보기 위하여 배추(*Brassica rapa* L.)에 형질전환하는 실험을 진행하였다. 전체 T1 형질전환체의 약 50%인

30 개의 형질전환 배추에서 웅성불임을 보였다. 그럼에도 불구하고 *orf456* 유전자가 웅성불임을 유도하는 정확한 기작은 밝힐 수 없었다.

주요어: 고추(*Capsicum chinense)*, 배추(*Brassica rapa* subsp. pekinensis), 세포질적 웅성불임, 유전자 지도, 돌연변이, 저온민감성.

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