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DNA-FREE TRANSCRIPTIONAL ACTIVATION OF CABBAGE (BRASSICA OLERACEA L.) USING CRISPR/DCAS9 RIBONUCLEOPROTEINS TO ENHANCE HEAT STRESS TOLERANCE BASED ON MORPHOPHYSIOLOGICAL PLANT TRAITS

MAHDI MORADPOUR

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By

MAHDI MORADPOUR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

March 2021

DEDICATION

To my dearest MOTHER and FATHER, for all their supports in this long journey.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Red cabbage (*B. oleracea*) is one of the most distinct species among the numerous species of *Brassica* genus due to having high level of anthocyanins. *Brassica* species are widely consumed vegetable crops with great health benefits. However, they are highly vulnerable to high temperature and their production are limited to highland areas in Malaysia. Understanding how plants adjust their developmental programs in response to temperature variations is central to sustain crop productivity in the modern agriculture facing global climate change. Global climate change has generated significant fluctuations of ambient growth temperature, which can profoundly influence diverse developmental, physiological, and morphological responses, including modulations in plant growth and yield. In recent years, many crop genomes have been sequenced and innovative biotechnological approaches allowed to take a step forward towards the development of new improved cultivars harboring precise genome modifications. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 system, represents the main methods available for plant genome engineering through targeted modifications. Such technology, however, requires efficient transformation protocols and extensive genomic resources and accurate knowledge before they can be efficiently exploited in practical breeding programs. This study investigated on heat-tolerant/sensitive cultivars based on morphophysiological indicators and the action and interaction of different genes in the molecular network to serve as critical tools for genetic improvement in cabbage. The feasibility of DNA-free transcriptional activation method through delivery of CRISPR/dCas9-based transcriptional activation domains (TADs) ribonucleoproteins (RNPs) into the red cabbage protoplasts was also evaluated. To screen the morphophysiological indicators, the morphological and physiological performance of two different varieties of white and red cabbages (*B. oleracea* var. *capitate* f. *alba* and f. *rubra*, respectively) under heat stress (HS) at 42°C for 5h and non-stress (NS) was evaluated. Cultivars that showed considerable cell membrane thermostability and less reduction on chlorophyll content

with better head formation were categorized as the heat tolerant cultivars (HTC). While those with reduction in stomatal conductance and higher reduction incurred on chlorophyll and damage on thylakoid membranes as the heat sensitive cultivars (HSC). In order to select the target genes, the expression of four key genes in cabbage HS response pathway were evaluated in HTCs and HSCs as determined by morphophysiological indicators. Expression profiles of key genes in HS response network including *BoHSP70* (HEAT SHOCK PROTEIN 70), *BoSCL13* (SCARECROW-LIKE 13), *BoDPB3* (transcriptional regulator DNA POLYMERASE II SUBUNIT B3 (DPB3))/NUCLEAR FACTOR Y SUBUNIT C10 (NF-YC10) evaluated in all cultivars under HS at 42ºC for 3h and 5h compared to NS. Based on the results, the morphophysiological and molecular indicators are applicable to cabbage cultivars for differentiating HTC and HSC and potential target genes for genome editing identified for enhancing food security in the warmer world. The results of expression profiling of these key genes in HS response network indicated that in order to increase tolerance of red cabbage to HS, *BoDPB3*, is a potential target gene for activation. A versatile protoplast system for delivery of RNPs composed of purified dCas9 fusion proteins fused to transcriptional activation domain (VP64) and four different *in vitro* transcribed (IVT) single guide RNAs (sgRNAs) using PEG into the red cabbage protoplasts was successfully established. The highest endogenous gene activation was 15.7-fold using RNP 3, for *BoDPB3* whereas RNP 3 and RNP1 modestly activated *BoDPB3* by 6.7-fold and by 4.6-fold, respectively indicating that the closer position of the sgRNAs targeting antisense and sense strands of *BoDPB3* promoter region to the transcriptional start site gave higher expression. The interaction of targeted HS responsive gene activation by CRISPR/dCas9-VP64-RNPs within HS regulatory network in red cabbage was also evaluated. The results showed that the dCas9-VP64 tool was capable to regulate transcriptional regulatory network of HS response genes through enhancing the expression *BoDPB3* by 15.7-fold which consequently led to significant suppression of the expression level of *BoDRIP* by 13.3-fold. Our study demonstrated that CRISPR/(d)Cas9-TAD RNPs is a potential tool for targeted gene activation, while avoiding the undesirable integration effects of plasmid DNA in the host genome. The CRISPR/(d)Cas9-TAD RNPs could be a valuable system for facilitating plant researchers in interrogating gene functions and for manipulating biological traits. Two HTC showed better head formation compared to cabbages from HSC. One HTC (WCC1) and two HSC (WCC3 and RCC) were selected to study the effects of HS on expression profiles of key genes in HS response network. Strong induced expression of HEAT SHOCK PROTEIN 70 (*BoHSP70*) was observed for all three cultivars under HS (RCC by 70.7-fold, WCC1 by 23.5-fold and WCC4 by 15.70-fold) compared to non-stress (NS) plants. *BoSCL13* which showed strong induced expression in WCC1 by 14.4-fold but not in WCC3 under HS can be used to differentiate HTC from HSC in green cabbage cultivars. *BoDPB3*, a DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A) interactor showed suppressed expression in RCC by 0.3-fold unlike WCC1 by 4.3-fold and WCC3 by 3.5-fold that demonstrated enhanced expression under HS, explaining the high sensitivity of RCC to HS.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGAKTIFAN TRANSKRIPSI BEBAS-DNA DALAM KUBIS (*BRASSICA OLERACEA* L.) MENGGUNAKAN RIBONUKLEOPROTEIN UNTUK MENINGKATKAN TOLERANSI TERHADAP TEKANAN HABA BERASASKAN CIRI MORFOFISIOLOGI TUMBUHAN

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Kubis merah (*B. oleracea*) adalah spesis paling menonjol di kalangan kebanyakan spesis daripada genus Brassica kerana ia mempunyai paras antosianin yang tinggi. Spesis Brassica adalah tanaman sayur yang dimakan dengan meluas dengan manfaat kesihatan yang besar. Bagaimanapun, ia sangat mudah terancam oleh suhu yang tinggi dan pengeluarannya terhad kepada kawasan tanah tinggi di Malaysia. Kefahaman terhadap bagaimana tumbuhan bertindak balas kepada perubahan suhu penting untuk memastikan kelestarian produktiviti pertanian moden dalam menghadapi perubahan iklim. Perubahan iklim global telah menyebabkan turun naik ketara suhu pertumbuhan ambien yang memberi kesan mendalam pelbagai tindak balas pengembangan, fisiologikal dan morfologikal termasuk perubahan dalam pertumbuhan dan hasil. Beberapa tahun kebelakangan ini, banyak genom tanaman telah dijujukkan dan pendekatan bioteknologi yang inovatif membolehkan langkah kehadapan kearah membangunkan kultivar baharu yang telan ditambah baik mengandungi genom yang terubahsuai secara terperinci. Sistem kluster palidromik pendek berulang berjarak teratur (CRISPR)/protein 9 berkaitan CRISPR merupakan kaedah utama yang ada untuk kejuruteraan genom tumbuhan melalui pengubahan tersasar. Teknologi sebegini bagaimanapun memerlukan protokol transformasi yang efisien dan sumber genomik yang meluas dan pengetahuan tepat sebelum boleh dieksploitasi dalam program pembiakbakaan yang praktikal. Kajian ini meneliti kultivar toleran/sensitif berasaskan indikator morfofisiologikal dan aksi juga interaksi gen berlainan dalam jaringan molekular yang berperanan sebagai peralatan kritikal untuk penambahbaikan genetik dalam kubis. Kebolehlaksanaan kaedah pengaktifan transkripsi bebas DNA melalui penghantaran ribonukleoprotein (RNPs) domain pengaktifan transkripsi (TADs) berasaskan CRISPR/dCas9 ke dalam protoplas telah di nilai. Untuk skrin indikator morfofisiologikal, prestasi dua varieti kubis putih dan merah (masing-masing *B. oleracea* var. *capitate* f. *alba* dan f. *rubra*) di bawah tekanan haba (HS) pada 42°C selama 5h dan tanpa tekanan (NS) telah dikaji. Kultivar yang menunjukkan kestabilantermo sel membran yang tinggi dan kurang penurunan kandungan klorofil dengan pembentukan kepala yang lebih baik dikategorikan sebagai

kultivar toleran haba (HTC). Manakala yang menurun konduktan stomatanya, lebih tinggi kerosakan membran tilakoid dan penurunan klorofil sebagai sensitif haba (HSC). Untuk memilih gen sasaran, ekspresi empat gen utama dalam rangkaian tindak balas HS kubis dinilai dalam HTCs dan HSCs berasaskan indikator morfofisiologi. Profil pengekspresan gen utama dalam jaringan tindak balas HS termasuk *BoHSP70* (HEAT SHOCK PROTEIN 70), *BoSCL13* (SCARECROW-LIKE 13), *BoDPB3* (transcriptional regulator DNA POLYMERASE II SUBUNIT B3 (DPB3))/NUCLEAR FACTOR Y SUBUNIT C10 (NF-YC10) dikaji dalam kesemua kultivar kubis di bawah HS pada 42ºC selama 3h dan 5h berbanding NS. Berasaskan penemuan indikator morfofisiologikal dan molecular tersebut boleh diguna pakai untuk kultivar kubis untuk membezakan HTC dan HSC dan gen berpotensi sebagai sasaran untuk penyuntingan genom dikenalpasti untuk meningkatkan sekuriti makanan dalam dunia yang lebih panas. Hasil penemuan pemprofilan pengekspresan gen utama dalam jaringan tindakbalas HS menunjukkan untuk meningkatakan toleran kubis merah kepada HS, *BoDPB3* adalah sasaran berpotensi untuk diaktifkan. Suatu sistem protoplas yang serba guna untuk penghantaran RNPs terdiri daripada protein dCas9 rekombinan tulen yang bergabung dengan domain pengaktifan transkripsi (VP64) dan empat RNA panduan tunggal (sgRNAs) tertranskrip berbeza menggunakan PEG ke dalam protoplas kubis merah telah berjaya dibangunkankan. Pengaktifan gen endogenus tertinggi diperolehi adalah sebanyak 15.7 kali ganda menggunakan RNP 3, untuk *BoDPB3* manakala RNP 3 dan RNP 1 mengaktifkan *BoDPB3* secara sederhana masing-masing sebanyak 6.7 dan 4.6 kali ganda, menunjukkan kedudukan sgRNAs yang lebih dekat mensasar bebenang antisense dan bebenang sense kawasan promoter *BoDPB3* dari tapak permulaan transkripsi memberi ekspresi yang lebih tinggi. Interaksi pengaktifan gen responsif HS tersasar oleh CRISPR/dCas9-VP64-RNPs dalam rangkaian kawal selia HS dalam kubis merah juga dinilai. Keputusan menunjukkan dCas9-VP64 mampu mengawal rangkaian kawal selia transkripsi bagi gen tindak balas HS dengan meningkatkan ekspresi *BoDPB3* sebanyak 15.7 kali ganda yang seterusnya menyebabkan penurunan ketara tahap ekspresi *BoDRIP* sebanyak 13.3 kali ganda. Kajian kami menunjukkan CRISPR/(d)Cas9-TAD RNPs adalah alat berpotensi untuk pengaktifan gen bersasar, di samping mengelakkan kesan persepaduan yang tidak diingini oleh plasmid DNA di dalam genom hos. CRISPR/(d)Cas9-TAD RNPs boleh menjadi sistem yang bernilai bagi memudahkan penyelidik tumbuhan dalam menginterogasi fungsi gen dan untuk memanipulasi ciri biologi. Dua HTC menunjukkan pembentukan kepala yang lebih baik berbanding kubis dari HSC. Satu HTC (WCCI) dan dua HSC (WCC3 dan RCC) telah dipilih untuk mengkaji kesan HS ke atas profil ekspresi gen utama dalam rangkaian tindak balas HS. Ekspresi terangsang tertinggi HEAT SHOCK PROTEIN 70 (*BoHSP70*) telah dikesan untuk semua tiga kultivar di bawah HS (RCC sebanyak 70.7 kali ganda, WCC1 sebanyak 23.5 kali ganda dan WCC4 sebanyak 15.70 kali ganda) berbanding tumbuhan tanpa tekanan (NS). *BoSCL13* yang menunjukkan ekspresi teransang yang kuat dalam WCC1 sebanyak 14.4 kali ganda tetapi tidak dalam WCC3 di bawah HS boleh digunakan untuk membezakan HTC dari HSC dalam kultivar kubis hijau. *BoDPB3*, interaktor DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A) menunjukkan ekspresi yang menurun dalam RCC sebanyak 0.3 kali ganda tidak seperti WCC1 sebanyak 4.3 kali ganda dan WCC3 sebanyak 3.5 kali ganda yang menunjukkan peningkatan ekspresi di bawah HS, menjelaskan mengenai sensistiviti RCC yang tinggi terhadap HS.

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This is to confirm that:

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A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
ANOVA	Analysis of Variance
AP2	APETALA2
B. oleracea	*Brassica oleracea*
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool Nucleotide
bp	Base pair
C	Control
Cas9	CRISPR associated protein 9
cDNA	Complementary DNA
CDS	Coding Region
CEC 1 and TEC 1	Initial conductance measurement
CEC 2 and TEC 2	Final conductance measurements
CF	Chlorophyll fluorescence
cm	Centimeter
CM1000™	Chlorophyll index values
CMCC	CM 1000 relative chlorophyll value
CMT	Cell membrane thermostability
CO2	Carbon dioxide
CRD	Completely Randomized Design
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference

crRNA	CRISPR RNA
CTAB	Hexacetyltrimethyl Ammonium Bromide
dCas9	Deactivated CRISPR-associated Protein 9/ Nuclease-deficent Cas9
dCas9-TAD	Deactivated CRISPR-associated Protein 9-Transcriptional Activation Domain
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribo Nucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DPB3	Transcriptional regulator DNA POLYMERASE II SUBUNIT B3 (DPB3))/NUCLEAR FACTOR Y SUBUNIT C10 (NF-YC10)
DRE	DNA replication-related element
DREB2A	DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN 2A
DSB	Double-Stranded Break
E. coli	*Escherichia coli*
EDLL	The conserved glutamic acid (E), aspartic acid (D) and leucine (L) residues
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
FIS2	Fertilization-Independent Seed2
Fm	Maximal fluorescence
Fo	Minimal fluorescence
Fv/Fm	Photosynthetic efficiency, the maximum quantum yield of PS II photochemistry
g	Gravity
Gb	Giga bite

GMOs	genetically modified organisms
gRNA	guide RNA
gs	leaf stomatal conductance
h	Hour
H2O2	Hydrogen Peroxide
HDR	Homology-Directed Repair
HNH domain	An endonuclease domain named for characteristic histidine and asparagine residues
HS	Heat stress
HSC	heat sensitive cultivars
HSD	Tukey's honestly significant difference Studentized Range Test
HSF	Heat shock factors
HSFA1	Heat shock transcription factor A1
HSP	Heat shock proteins
HSP100	Heat shock protein 100
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
HT	High Temperature
HTC	heat tolerant cultivars
IPCC	Intergovernmental Panel on Climate Change
IVT	*In vitro* Transcription
IVT-sgRNA	*In vitro* transcribed single guide RNA
kb	Kilo Base-Pair
Kb	Kilobase pair
Km	Michaelis constant
KRAB	Kruppel-associated Box

L	Liter
LB	Luria-Bertani
LiCl	Lithium Chloride
LUC	luciferase reporter gene
M	Molar
mg	Miligram
min	Minute
mL	Milliliter
mM	Milimolar
mm	Millimeter
mm^2	Square millimeter
mmol m^{-2} s^{-1}	Measurement unit of stomatal conductance and transpiration
mRNA	Messenger RNA
MYB	Myeloblastosis gene
NaCl	Sodium Chloride
NaOH	Natrium Hydroxide
NCBI	National Center For Biotechnology Information
ng	Nanogram
NGS	Next-generation sequencing
nm	Nanometer
OD	Optical Density
PAM	Protospacer-Adjacent Motif
PAP1	Production of Anthocyanin Pigment1
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
pH	Potential Hydrogen

PIABS	performance index
PSII	Photosystem II
PVP	Polyvinylpyrrolidone
qPCR	Quantitative real-time PCR
RCC	Red Cabbage Cultivar
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
ROS	reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Rubisco	ribulose-1, 5-bisphosphate carboxylase/oxygenase
RuvC domain	An endonuclease domain named for an E. coli protein involved in DNA repair
s	Second
S. pyogenes	*Streptococcus pyogenes*
S.O.C	Super Optimal Broth
SAS	Statistical Analysis System
SDS	Sodium Dodecyl Sulphate
SE	Standard Error
sgRNA	single guide RNA
T	Treatment
TAD	Transcriptional Activation Domain
TAE	Tris-acetate EDTA
TALE	transcription activator-like effector

TALEN	Transcription activator-like effectors proteins
TATA-box	Goldberg-Hogness box
T-DNA	transfer DNA
TE buffer	Tris-EDTA buffer
TE	Tris EDTA
Tm	Melting temperature
TMD	thylakoid membrane damage
tracrRNA, trRNA	Trans-activating crRNA
Tris-HCl	Trisaminomethane hydrochloride
UPM	Universiti Putra Malaysia
UTR	Untranslated region
UV	Ultra Violet
V	Volt
v/v	volume/volume
VP16	Herpes simplex viral protein 16
w/v	weight/volume
WCC1	White Cabbage Cultivar 1
WCC2	White Cabbage Cultivar 2
WCC3	White Cabbage Cultivar 3
WCC4	White Cabbage Cultivar 4
ZnF	Zinc-finger proteins
µE m−2s−1	Microeinsteins per second per square meter
E	The einstein (symbol E) is a unit defined as the energy in one mole of photons (6.022×1023 photons)
°C	Degrees Celsius
µL	Microliter

µm	Micrometer
µmol m^{-2} s^{-1}	Micromole per second and square meter
µmole	Micromole

CHAPTER 1

INTRODUCTION

Malaysia imports around 880,000 tons of vegetables annually from USA, Holland and China Thailand, mainly temperate type of produce like cabbage, carrot and cauliflower. The demand for vegetable in Malaysia is increasing from 1.91 million tons in 2015 to 2.4 million tons in 2020 with growth rate of 4.5% per annum (Halim & Rozhan, 2018). However, in Malaysia due to the high temperature and humid climate, production of cabbage is almost impossible in the lowlands. Cameron Highlands is the focus area for its cultivation (Jusoff, 2010). Hence, there is great potential in growing heat tolerant cultivars of cabbages in the lowlands of Malaysia due to the high market demand and limited highland area.

Red cabbage (*Brassica oleracea*, var. *capitata*) is a very popular edible cabbage with slightly sweet taste, characterized by the beautiful purple- and magenta-colored leaves due to anthocyanin accumulation. Anthocyanin is a large group of water-soluble pigments that are usually distributed in higher plants. It belongs to the flavonoid group of compounds which play important role to protect plants against various abiotic and biotic stresses (Yuan *et al.,* 2009). Red cabbage like other *Brassica* vegetables is of low-calorie and serve as a rich source of glucosinolates and carotenoids, besides other vitamins, minerals and anthocyanins that has benefits for human health (Park *et al.,* 2013; Ravanfar *et al.,* 2017). However, *Brassica* vegetables are cool season crops and most varieties are highly vulnerable to high temperature which stimulates various and often incompatible changes in plant growth, development and physiological processes which together adversely affecting crop yield (Hasanuzzaman *et al.,* 2013b). Moreover, many reports have shown that crop productivity will be negatively affected even by small increment in temperature. The main goal of the 2015 Paris Climate Agreement is to limit future global warming to less than 2.0°C above pre-industrial levels. The Intergovernmental Panel on Climate Change (IPCC) Special Report expected that global warming is likely to reach 1.5°C between 2030 and 2050 if it continues to increase at the current rate (Masson-Delmotte, 2018). Hence, adaptation to climate change and higher temperature is one of the most important challenges for crop production in many parts of the world as well as in Malaysia.

Research on response of vegetable crops to heat stress (HS) is critical due to climate change effects on crop production. Thus, improving heat tolerance of commonly consumed crops has become an important breeding goal. Genetic improvement of plants to withstand HS is a key strategy to accede to this goal. The conventional plant breeding methods work well for breeding resistance against a number of abiotic and biotic stresses in vegetable crops. But there are limited achievements reported on heat stress due to the complexity of the biochemical/physiological mechanisms involved in heat stress response. Similarly, through marker-assisted breeding there were several successes in improving abiotic stresses for example salt stress (Singh *et al.,* 2018) and flooding stress (Sandhu *et al.,* 2019) but not on HS. The transgenic approach emerges as a great tool for

addressing several agronomic traits like breeding to improve resistance against stresses. However, once plants have been genetically modified using *Agrobacterium* tumefaciens resulting in the insertion of foreign gene, such as an antibiotic selection marker, the plants are legally designated as genetically modified organisms (GMOs). They will be subjected to strict regulatory procedures before commercialization (Choe, 2016).

During the past two decades, many heat-related genes including heat shock proteins (HSPs), heat shock factors (HSFs) and heat stress inducible genes have been successfully cloned, and their roles on specific metabolic activities in governing the plant heat response have been studied. Among them, DPB3 is an interactor with the transcription factor DREB2A. It has been reported that the overexpression of DPB3 improves HS tolerance in *Arabidopsis* and rice by increasing the expression of various stress-inducible genes under HS conditions (Sato *et al.,* 2016; Ohama *et al.*, 2017; Su *et al.*, 2019). In our study also, it was hypothesized that the expression of DPB3 as master regulator gene in heat stress-response pathways improves HS tolerance in cabbage as well. Hence, understanding the morphophysiological and molecular responses to HS among HTCs and HSCs in *Brassica oleracea* L. species would be the key in this hypothesis.

Overexpression of complementary DNA (cDNA) is a promising approach for gene functional studies and manipulation of biological traits. However, this approach is challenging and unproductive for expression of multigene due to being tedious for cloning, requirement of multiple promoters and terminators, limitation of vector capacity, and inconstant transgene expression levels (Li *et al.,* 2017; Moradpour & Abdulah, 2017). Artificial transcriptional activators offer likely an alternative strategy for gene activation by implementing transcription activation domain (TAD) to a targeted gene promoter at the endogenous genomic locus by a programmable DNA-binding module (Li *et al.,* 2017). The nuclease-dead *Streptococcus pyogenes* Cas9 (dCas9) protein is one of the DNA-binding modules that is capable to recognize a specific DNA target through base pairing between an artificial guide RNA and DNA is a distinctive genetic tool for targeted gene regulation. It outperforms other gene editing tools including zinc-finger (ZnF) proteins and transcription activator-like effectors (TALEN) in terms of efficiency, precision and versatility (Qi *et al.*, 2013). Recently, a few powerful dCas9-based gene activation systems have been developed for plant cells. However, an effective dCas9-based transcriptional activation platform is still required for plant cells to improve the level of expression (Piatek *et al.,* 2015; Lowder *et al.,* 2015; Vazquez-Vilar *et al.*, 2016; Li *et al.,* 2017).

Massive genome and transcriptome databases available serve as valuable genomic resources for crop improvement through targeted gene regulation. Although CRISPR/(d)Cas9 systems alterations are similar with naturally occurring mutations, the practice of transgenic system during development of specified varieties still causes GMO legislation in countries that depend on process-based regulation (Murovec *et al.,* 2018), meaning that all organisms produced by genetic engineering must be approved by the regulatory system prior to release.

Furthermore, insertional mutagenesis is a consequence of stably integration of DNA coding region into plant genomes using CRISPR tools, but its prolonged expression led

to mutations in off-target sites. These consequences can be avoidable with the delivery of RNP complexes that composed of purified recombinant enzyme (d)Cas9 and IVT-sgRNA. Because this dead version of Cas9 does not have capability to cleave DNA, but dCas9 still is capable to target and bind to DNA with the same accuracy when directed by sgRNA. On the other hand, instead of irreversibly alteration of the genome, binding of dCas9 intervenes with transcription at the target site, causing in reversible activation or suppression of the gene (Moradpour & Abdulah, 2020). Hence, the main aims of this study were:

1) To determine a master regulator gene in heat stress-response pathways through evaluating morphophysiological and molecular responses to HS among HTCs and HSCs in *Brassica oleracea* L. species

2) To produce a purified recombinant dCas9-based transcriptional activation domain (dCas9-TADs) protein and *in vitro* transcribe single guide RNAs (IVT-sgRNAs) from different promoter positions of the targeted gene

3) To develop protoplast isolation protocol of red cabbage and transfect the protoplasts with RNP complexes consisting of dCas9-TAD combine with the different IVT-sgRNAs

4) To analyse the transcriptional activation by RT-qPCR of the target gene in the transfected protoplasts and evaluate the effects of different sgRNA positions on the transcriptional activation efficiencies

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