EMPLOYING CRISPR_CAS9 (Developing new methods and knocking out phosphatase related genes in Tomato and Arabidopsis)

Contents

1.1 Introduction about CRISPR-CAS9:

Programmable nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA guided endonucleases (RGENs) can facilitate genome-editing targeting by increasing the efficiency of homologous recombination. Recently, the newly discovered RGENs: clustered regularly interspaced short palindromic repeats (CRISPRs) is overcoming ZFNs and TALENs that depend on the time-consuming design and difficulties of their optimization. Bacteria and archaea have evolved adaptive immune defenses termed CRISPR–associated (Cas) systems that use short RNA to direct degradation of foreign nucleic acids (Figs 1, 2).

Type II CRISPR-Cas systems have been engineered to effect robust RNA-guided genome modifications in multiple eukaryotic systems. Different Cas9 variants have been engineered to increase the applicability of the CRISPR-Cas9 system. Cas9 has two active sites, and each site is responsible for cutting one of the complementary DNA strands. The double-strand break can either be repaired by the non-homologous end-joining (NHEJ) or homology directed repair (HDR). The error-prone NHEJ is the common pathway and usually induces small deletion or insertion mutations, or point mutation. The balance between NHEJ and HDR can be slightly shifted towards HDR by providing a donor template. By including a transgene within the donor template, gene targeting can be achieved.

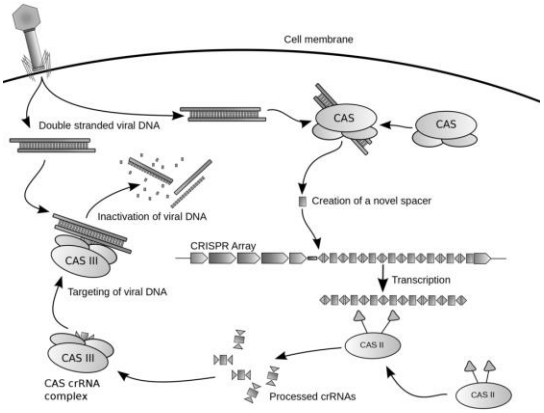


Fig. 1 Diagram of the CRISPR prokaryotic viral defense mechanism *Horvath P, Barrangou R (2010).*

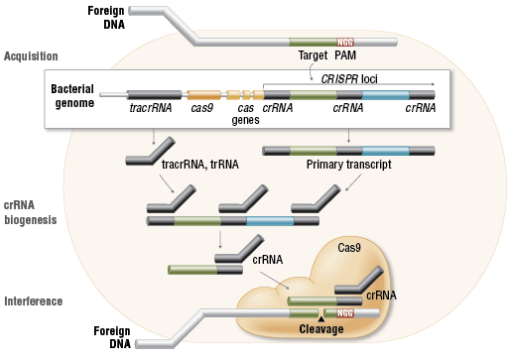


Fig. 2 Diagram of the CRISPR prokaryotic defense mechanism (NEB website)

1.2 Protein phoshphatases (PP4 regulators):

Protein phosphorylation and dephosphorylation represent essential regulatory mechanisms of reversible post-translational modification that controls many cellular processes. Protein kinases transfer the γ-phosphate group from ATP to the hydroxyl group of Ser, Thr and Tyr residues, whereas phosphatases hydrolyze the phosphoester bond to dephosphorylate proteins (Uhrig et al. 2013; Lillo et al. 2014). Arabidopsis has around 1125 protein kinases and 150 protein phosphatases. However, because of the many regulatory subunits involved in phosphatase complexes, it is believed that phosphatases can rival kinases according to substrate specificity (Brautigan 2013; Lillo et al. 2014).

Protein phosphatase 4 (PP4) is remarkably well conserved across eukaryotes. A. thaliana has two PP4 catalytic subunits, namely PP4-1 (At4G26720) and PP4-2 (At5g55260). In addition to these two catalytic subunits, the active PP4 complex contains also two subunits with regulatory functions, PP4R2L (At5g17070) and PSY2L (At3g06670). In mammals, PP4 is implicated in many biological process such as apoptosis regulation (Mourtada-Maarabouni and Williams, 2008), microtubule organization (Han et al., 2009), and recovery from DNA damage checkpoint (Nakada et al., 2008). PP4 also plays a non-redundant role for the differentiation, suppressor activity and gut homeostatis of Treg cells (Liao et al., 2014). Semi-lethal phenotype was produced by disrupting PP4 gene in Drosophila melanogaster (Helps et al., 1998). Depletion of PP4 gene in Caenorhabditis elegans by RNA-mediated interference (RNAi) showed aberration in formation of spindle in both mitosis and sperm meiosis (Sumiyoshi et al., 2002). Conditional knock out PP4 in mice T-cell inhibited the development of T-cells (Shui et al., 2007). However, recent study by Huang et al. (2016) shows that both up-regulation and inhibition of PP4 inhibited cell proliferation in HepG2 cells, indicated that PP4 plays dual roles during cell proliferation. (From Toga's Master thesis 2016)

Putative regulatory proteins for PP4 in A. thaliana were bioinformatically detected, namely PP4R2L (At5G17070) and PSY2L (At3G06670). SMK-1, putative homolog of PSY2L in C. elegans, is reported to promote longevity by modulating DAF-16 (FOXO transcriptional factor) without affecting other processes regulated by IIS or Insulin/IGF-1 signaling (Wolff et al., 2006). The functional homolog of PSY2L in Saccharomyces cerevisiae, PSY2, in coordination with other protein, plays role in the DNA damage response (O'Neill et al., 2004). Moreover, in Homo sapiens, Ppp4R3 in complex with Ppp4c and PP4R2 involved in anticancer cisplatin sensitivity, linked to the DNA damage response (Gingras et al., 2005). The functional homolog of PPR2K in Saccharomyces cerevisiae, YBL1046W, binds the catalytic subunits PPH3 and also shows resistance to cisplatin (Hastie et al., 2006). (From Toga's Master thesis 2016). From our research, PSY2L knockdown and out by T-DNA insertions and RNAi approach, produce dwarf and noticeable phenotypes on Arabidopsis thaliana plants.

1.3 Peroxisomal phosphatases:

Peroxisomes are important eukaryotic organelles that fulfill several metabolic functions such as anabolic and catabolic lipid metabolism, free radical detoxification, development, and stress-related functions. Peroxisome disorders have a strong impact on plant development and sometimes fatal in mammals. However, research on peroxisome biogenesis and functions has been intensified, but their control by phosphoregulation is barely studied. Remarkably, we identified the first-known peroxisomal protein phosphatase (PP2A-B'theta heterotrimeric complex), and show its positive impact on fatty acids beta-oxidation in the model plant Arabidopsis thaliana (Kataya et al. 2015a). We also consolidate evidences that the mitochondrial regulatory subunit of PP2A (B'zeta) has a role in energy metabolism and salt stress. In the same context, we are focusing on perceiving the yet uncharacterized peroxisome phosphoregulation. To embark on plants, we searched Arabidopsis genome for phosphatase-related proteins that harbors putative peroxisomal signals and experimentally validated additional four peroxisomal protein phosphatases (two PP2C, one MAPK phosphatase, and one purple acid phosphatase) (Kataya et al. 2015b, 2016). In order to understand the impact of the newly identified phosphatases on peroxisomes functions and plant development, we need to obtain knockout lines of their genes using CRISPR/Cas.

2.1 Master projects:

We wish to use the new technology of CRISPER/Cas9 for editing specific genes for basic and applied research purposes. We wish to establish a method that allows generating free DNA-modified plants (GMOs) that can probably be more acceptable than traditionally known GMOs that have different leftover foreign DNAs from delivery techniques (see updated plant related reviews as (Graham and Root 2015; Ding et al. 2016; Khatodia et al. 2016). Successfully, this was reported using combination of CAS9 protein and gRNAs transcript and their subsequent transformation to plant protoplasts (Woo et al. 2015).

2.1.1 Project-1

Employing CRISPR-CAS9 approach for DNA free editing of *Solanum lycopersicum* genome with pre-assembled CRISPR-CAS9 ribonucleoproteins and transcripts

This project will investigate the ability to produce a DNA-free modified GMOs (Tomato) using CRISPR/CAS9 and tissue culture technology.

Methods: Molecular biology (cloning), protoplast production and transfection, In vitro transcription and mRNA purification, and Tissue culture methodology

1- Planting tomato seeds and make tissue culture propagation for tomato plants
2- Performing and establishing tissue culture system to re-generate plants from isolated protoplasts (Tomato)
3- Cloning of guide RNAs that can target one or more genes (see figures 3)
4- In vitro transcriptions for cloned gRNAs and CAS9
5- Performing in-vivo CRISPR/CAS9 genetic modifications in vivo through the PEG transfections of isolated protoplasts by CAS9 protein (and/or CAS9 transcripts) and transcribed gRNAs against the marker gene PDS
6- Optional: checking the level of CAS9 protein by western after protoplast transfection by CAS9 transcripts
7- Screening using phenotypes and confirm using various molecular biology tools for successful modified plants

2.1.2 Project-2

Investigating DNA-free genome editing in *Arabidopsis thaliana* with pre-assembled CRISPR-CAS9 ribonucleoproteins and transcripts

This project will investigate the ability to produce a DNA-free modified GMOs (Arabidopsis) using CRISPER/CAS9 and tissue culture technology.

Methods: Molecular biology (cloning), protoplast production and transfection, in vitro transcription and mRNA purification, protein production and purification, and Tissue culture methodology

1- Performing and establishing tissue culture system to re-generate plants from isolated protoplasts (Arabidopsis)
2- Expression and purification of NLS-CAS9 protein
3- Cloning of guide RNAs that can target one or more genes (see figures 3) and subsequent in-vitro transcription for gRNAs
4- In vitro transcriptions for cloned gRNAs and CAS9
5- Performing in-vivo CRISPR/CAS9 genetic modifications in vivo through the PEG transfections of isolated protoplasts by CAS9 protein and transcribed gRNAs against the marker gene PDS3
6- Optional: checking the level of CAS9 protein by western after protoplast transfection by CAS9 transcripts
7- Screening using phenotypes and confirm using various molecular biology tools for successful modified plants

2.1.3 Project-3

Targeted mutagenesis of peroxisomal protein phosphatases using CRISPR-CAS9 approach

Methods: Molecular biology (cloning), protoplast production and transfection, protein production and purification, and Agrobacterium transformation and generating of stable plants. Moreover, Tomato PSY2L will be knocked out using the same technology.

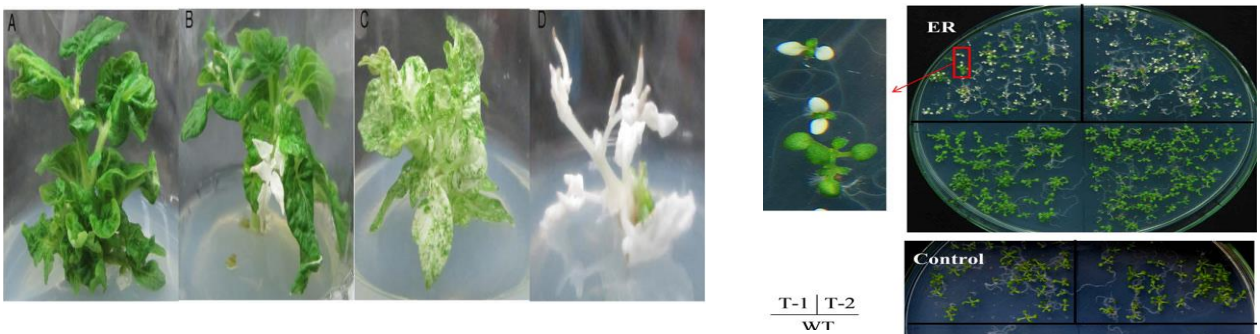
1- Cloning of guide RNAs that can target one or more genes (see figures 3) into binary vectors
2- Transforming the recombinant vectors into Agrobacterium
3- Performing in-vivo CRISPER/CAS9 genetic modifications in vivo through the PEG transfections of isolated protoplasts by recombinant vectors
4- Screening protoplasts for successful editing
5- Follow up on collecting seeds of stable transgenic plant and screening using phenotypes and selection markers and confirm using various molecular biology tools for successful modified plants
6- CRISPR/Cas9 editing of Tomato PSY2L by Agrobacterium transformation method

3.1 Tomato Solanum lycopersicum

CRISPR/Cas9 editing in Tomato has been done recently with two methods: using Agrobacterium based delivery (Brooks et al. 2014; Pan et al. 2016) or viral based delivery (Cermak et al. 2015) of CAS9 and sgRNAs. This technology is hoped for crop improvement by editing the plants to have strong immunity against infection (Chaparro-Garcia et al. 2015) for example, researchers were able to use CRISP/CAS9 to strengthen Nicotiana benthamiana immunity against DNA virus infection (Ali et al. 2015). If such ambitions can be applied using DNA-free editing in crops, this will help us to avoid major criticism and fears about using GMO's. So far, DNA-free editing using the important crop "tomato" not yet has been done, and we wish to establish the method that can help us achieve this target.

3.1.1 Utilizing PDS as a quick marker protein for reverse genetics approaches:

Phytoene desaturase (SlPDS, Solyc03g123760.2.1). SlPDS encodes phytoene desaturase, the key enzyme in carotenoid biosynthesis, and silencing the gene will cause photobleaching or albino phenotypes. Remarkably, 54.54% (12 out of 22) of the sgRNA1-SlPDS and 57.14% (4 out of 7) of the sgRNA2-SlPDS transgenic plants showed an albino phenotype, indicating the complete or partial loss of SlPDS function (Pan et al., 2016). This gene was also used as a marker for CRISPR/CAS9 in Nicotiana tabacum (Gao et al. 2015) and Maize (Feng et al. 2016). Also, PDS3 knock-down using RNAi technology produced the same phenotype (Jiang et al. 2013). In Arabidopsis, PDS3 was also used for CRISPR editing (Li et al. 2013).



Left figure: Albinism phenotype of SlPDS transgenic plants in T0 generation. (A) WT (contain T-DNA). (B,C) chimeric mutant. (D) biallelic mutant. Mutant B, C and D show albino phenotype to varying degrees. (Pan et al., 2016). Right Figure: Arabidopsis T_2 plants from transformation with pER8: PDSi-awere grown on media supplemented with 17b-estradiol (ER, top) and on standard media (control, bottom). Plants were photographed 7 days after transferring to the 17b-estradiol-containing media. T-1 and T-2 were two transgenic lines. Jiang Y et al., 2013

3.1.2 Identifying Spacers for sgRNA production to Edit Tomato PDS:

Spacers for sgRNA 1 and 2, from **Pan et al., 2016,** are also found in the result of the prediction from CRISPR plant (crispr.wustl.edu/). However, CRISPR plant predicted much stronger ahead of them…

Your query is: Solyc03g123760 in Chr3 from 64554061 to 64561664

Class0.0 gRNA						
SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr3:64554870-64554890:c	NA	4	TAGTTGGGCGCGGAGAAGCA	CGGAACGTTG	-	exon

gRNA (Spacer was shown in upper-case):
5'-TAGTTGGGCGCGGAGAAGCAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-TAGTTGGGCGCGGAGAAGCA-3'
5'-TGCTTCTCCGCGCCCAACTA-3'

GC content of Spacer sequence: 0.6
Potential Pol III terminator (TTTTT): null
2 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
AclI cut AACGTT
HpyCH4IV cut ACGT

Class1.0 gRNA_1

Chr3:64556691-64556711	3	4	TAACGATCGATTGCAATGGA	AGGAACATTC	+	exon

gRNA (Spacer was shown in upper-case):
5'-TAACGATCGATTGCAATGGAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-TAACGATCGATTGCAATGGA-3'
5'-TCCATTGCAATCGATCGTTA-3'

GC content of Spacer sequence: 0.4
Potential Pol III terminator (TTTTT): null
3 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
BsrDI cut GCAATG
Nb.BsrDI cut GCAATG
HpyCH4V cut TGCA

Class1.0 gRNA_2

Chr3:64557090-64557110	4	4	GGACTCTTGCCAGCAATGCT	TGGAGGGCAA	+	exon

gRNA (Spacer was shown in upper-case):
5'-GGACTCTTGCCAGCAATGCTgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

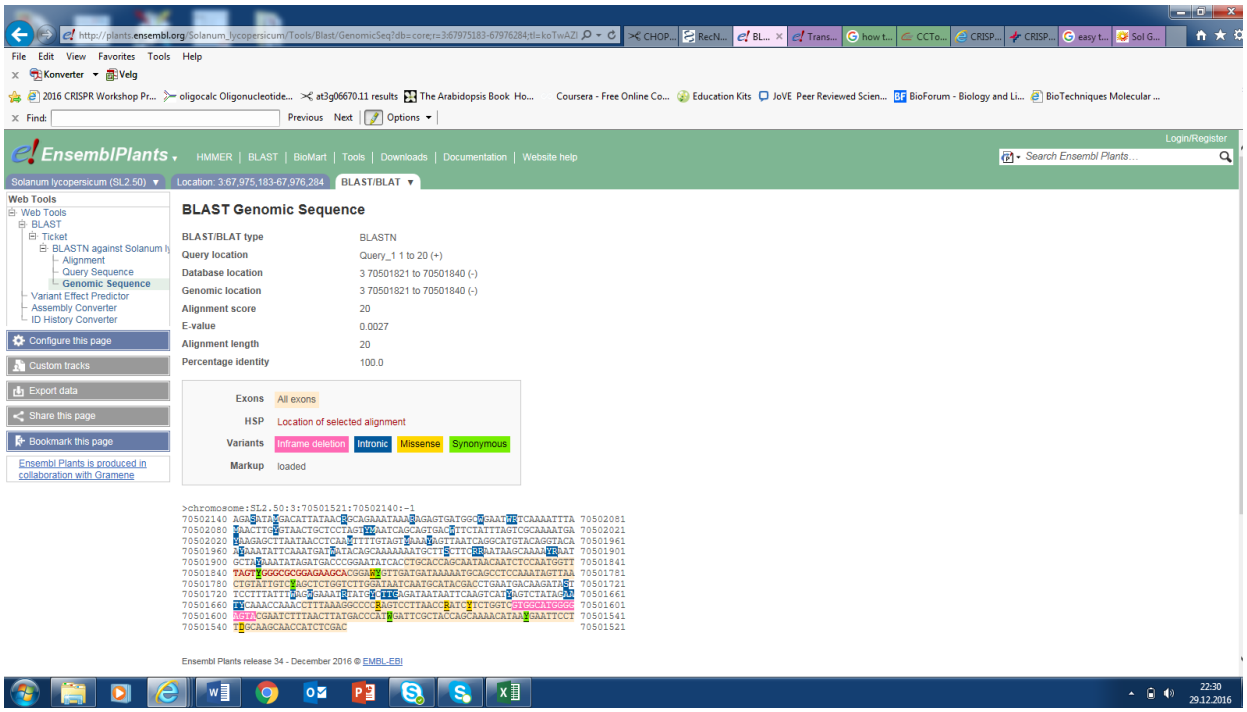
Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GGACTCTTGCCAGCAATGCT-3'
5'-AGCATTGCTGGCAAGAGTCC-3'

GC content of Spacer sequence: 0.55
Potential Pol III terminator (TTTTT): null
2 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
BsrDI cut GCAATG
Nb.BsrDI cut GCAATG

In order to find flanking primers for PCR amplification around the CRISPR target, I aligned the sGRNA against tomato genome in Ensembel, and found the target place… This will be used to design primers…

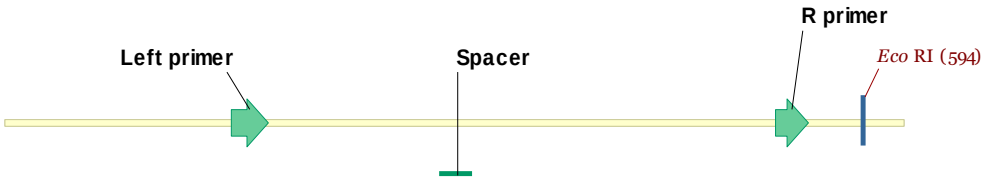
For the first spacer

BLAST Genomic Sequence




```
>chromosome:SL2.50:3:70501521:70502140:-1
70502140 AGASATAMGACATTATAACRGCAGAAATAAARAGAGTGATGGCWGAATWRTCAAAATTTA
70502081
70502080 MAACTTGYGTAACTGCTCCTAGTYMAATCAGCAGTGACWTTCTATTTAGTCGCAAAATGA
70502021
70502020 YAAGAGCTTAATAACCTCAAMTTTTGTAGTMAAAYAGTTAATCAGGCATGTACAGGTACA
70501961
70501960 AYAAATATTCAAATGATWATACAGCAAAAAAATGCTTSCTTCRRAATAAGCAAAAYRAAT
70501901
70501900 GCTAYAAATATAGATGACCCGGAATATCACCTGCACCAGCAATAACAATCTCCAATGGTT
70501841
70501840 TAGTYGGGCGCGGAGAAGCACGGAWYGTTGATGATAAAAATGCAGCCTCCAAATAGTTAA
70501781
70501780 CTGTATTGTCYAGCTCTGGTCTTGGATAATCAATGCATACGACCTGAATGACAAGATAST
70501721
70501720 TCCTTTATTTWAGWGAAATRTATGYCTTCAGATAATAATTCAAGTCATYAGTCTATAGAA
70501661
```

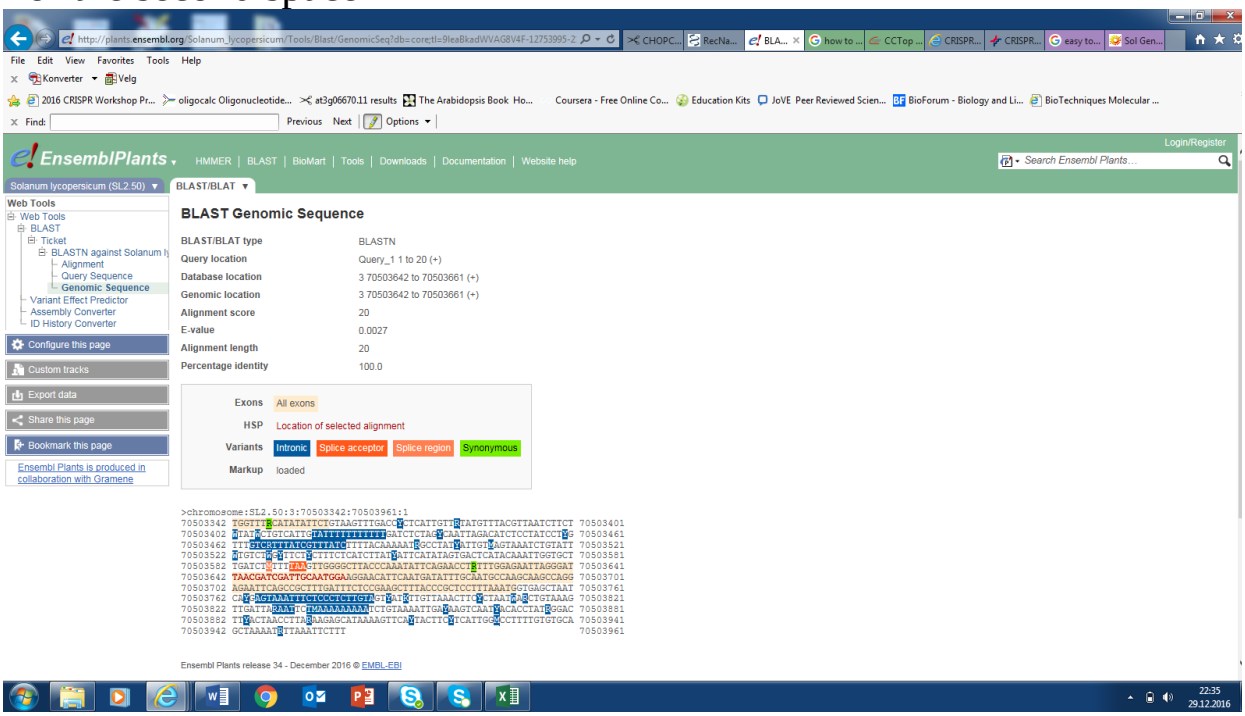
```
70501660 TYCAAACCAAACCTTTAAAGGCCCCRAGTCCTTAACCRATCYTCTGGTCGTGGCATGGGG
70501601
70501600 AGTACGAATCTTTAACTTATGACCCATWGATTCGCTACCAGCAAAACATAAYGAATTCCT
70501541
70501540 TDGCAAGCAACCATCTCGAC
70501521
```



SLPDS-SPACER!

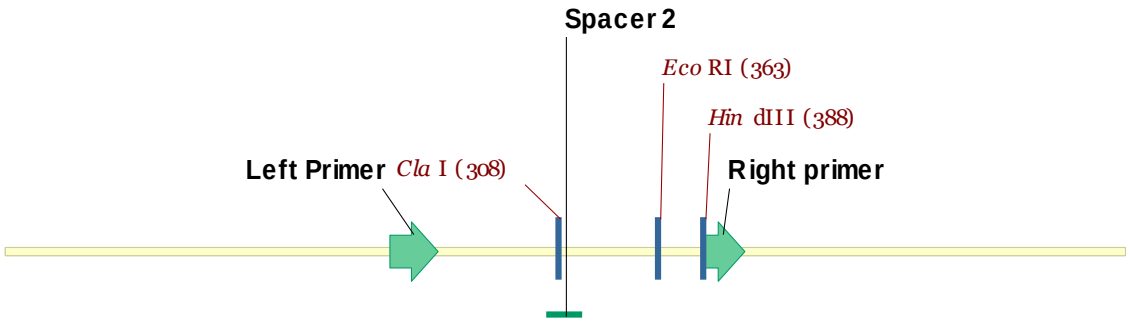
620 bp

For the second spacer




```
>chromosome:SL2.50:3:70503342:70503961:1
70503342 TGGTTTRCATATATTCTGTAAGTTTGACCYCTCATTGTTRTATGTTTACGTTAATCTTCT
70503401
70503402 WTATWCTGTCATTGTATTTTTTTTTTTTGATCTCTAGYCAATTAGACATCTCCTATCCTYG
70503461
70503462 TTTGTCRTTTATCGTTTATCTTTTACAAAAATRGCCTATYATTGTMAGTAAATCTGTATT
70503521
70503522 WTGTCTWGYTTCTYCTTTCTCATCTTATYATTCATATAGTGACTCATACAAATTGGTGCT
70503581
```

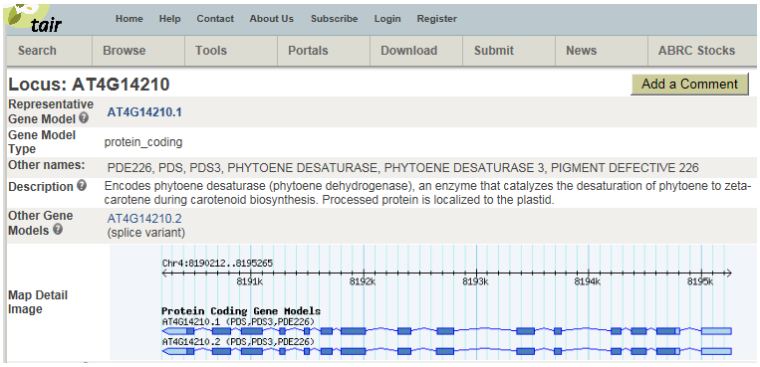
```
70503582 TGATCTMTTTTAAGTTGGGGCTTACCCAAATATTCAGAACCTRTTTGGAGAATTAGGGAT
70503641
70503642 TAACGATCGATTGCAATGGAAGGAACATTCAATGATATTTGCAATGCCAAGCAAGCCAGG
70503701
70503702 AGAATTCAGCCGCTTTGATTTCTCCGAAGCTTTACCCGCTCCTTTAAATGGTGAGCTAAT
70503761
70503762 CAYGAGTAAATTTCTCCCTCTTGTAGTYATKTTGTTAAACTTCYCTAATWARCTGTAAAG
70503821
70503822 TTGATTARAATTCTMAAAAAAAAATCTGTAAAATTGAYAAGTCAATYACACCTATRGGAC
70503881
70503882 TTYACTAACCTTARAAGAGCATAAAAGTTCAYTACTTCYTCATTGGMCCTTTTGTGTGCA
70503941
70503942 GCTAAAATRTTAAATTCTTT                                        70503
```



SLPDS1-Spacer 2

620 bp

3.2 Identifying Spacers for sgRNA production to Edit Arabidopsis PDS3:



Your query is: AT4g14210 in Chr4 from 8190212 to 8195265

Class0.0 gRNA						
SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location

Chr4:8192698-8192718	NA	NA	ATAAGCCTGACCGCCGACCA	TGGCTGGCAA	+	exon

gRNA (Spacer was shown in upper-case):
5'-ATAAGCCTGACCGCCGACCAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttt-3'

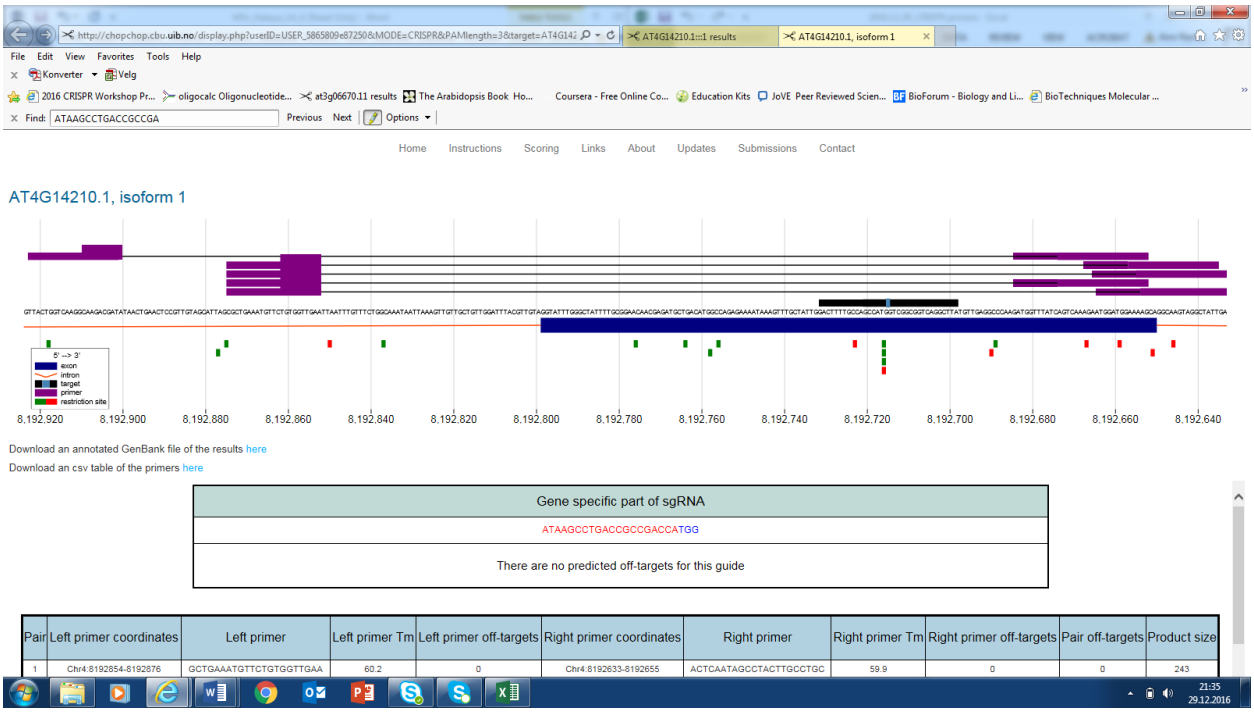
It is located in the middle of PDS3 mRNA:

Hit#	Sequence name	# of hits	Hit pattern	start	end	Hit sequence
1	AT4G14210.1	1	TGGTCGGCGGTCAGGCTTAT	1003	984	sequence
2	AT4G14210.2	1	TGGTCGGCGGTCAGGCTTAT	1003	984	sequence

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-ATAAGCCTGACCGCCGACCA-3'
5'-TGGTCGGCGGTCAGGCTTAT-3'

GC content of Spacer sequence: 0.6
Potential Pol III terminator (TTTTT): null
4 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
NlaIII cut CATG
CviAII cut CATG
FatI cut CATG
NcoI cut CCATGG

Total of 1 class0.0 gRNA seeds were found in this region



Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr4:8192854-8192876	GCTGAAATGTTCTGTGGTTGAA	60.2	0	Chr4:8192633-8192655	ACTCAATAGCCTACTTGCCTGC	59.9	0	0	243
2	Chr4:8192854-8192876	GCTGAAATGTTCTGTGGTTGAA	60.2	0	Chr4:8192653-8192675	GCTTTTCCATCCATTCTTTGAC	60.0	0	0	223
3	Chr4:8192854-8192876	GCTGAAATGTTCTGTGGTTGAA	60.2	0	Chr4:8192634-8192656	CTCAATAGCCTACTTGCCTGCT	60.1	0	0	242
4	Chr4:8192854-8192876	GCTGAAATGTTCTGTGGTTGAA	60.2	0	Chr4:8192636-8192658	CAATAGCCTACTTGCCTGCTTT	59.9	0	0	240
5	Chr4:8192902-8192924	TACTGGTCAAGGCAAGACGATA	59.8	0	Chr4:8192653-8192675	GCTTTTCCATCCATTCTTTGAC	60.0	0	0	

Chr4:8194628-8194648:c	3	4	CGCTTAAGACAAGAACAAGG	AGGAGGAGTA	-	exon

gRNA (Spacer was shown in upper-case):
5'-CGCTTAAGACAAGAACAAGGgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

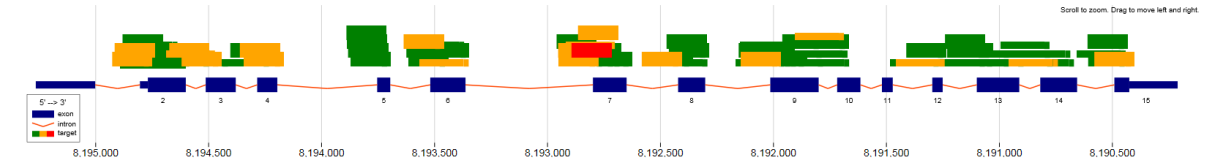
Hit#	Sequence name	# of hits	Hit pattern	start	end	Hit sequence
1	AT4G14210.1	1	CGCTTAAGACAAGAACAAGG	420	439	sequence
2	AT4G14210.2	1	CGCTTAAGACAAGAACAAGG	420	439	sequence

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-CGCTTAAGACAAGAACAAGG-3'
5'-CCTTGTTCTTGTCTTAAGCG-3'

GC content of Spacer sequence: 0.45
Potential Pol III terminator (TTTTT): null
1 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
BseRI cut GAGGAG

It is located in the beginning of PDS3 mRNA, also found from the website prediction ChopChop (see the next description):

AT4G14210.1, isoform 1

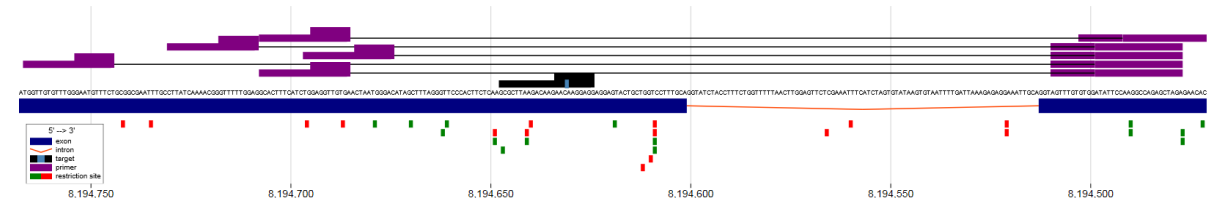


Download results: Results table

View in UCSC genome brows

Ranking	Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
							0	1	2	3	
1	TGGACCAAGAAAGTTGTCGGAGG	Chr4:8190446	15	-	52	1	0	0	0	0	0.73
2	GCTAGTATTTGCACCAGCAGAGG	Chr4:8191046	13	-	52	0	0	0	0	0	0.71
3	GCATGGTAGTAAACCTGGAGCGG	Chr4:8191787	9	+	57	0	0	0	0	0	0.70
4	GGCATACAAAGCCTTTCCGGAGG	Chr4:8191953	9	+	57	2	0	0	0	0	0.72
5	CGCTTAAGACAAGAACAAGGAGG	Chr4:8194626	2	-	48	0	0	0	0	0	0.70
6	CGGCGGTCAGGCTTATGTTGAGG	Chr4:8192692	7	-	61	0	0	0	0	0	0.64
7	AGAAAGGTAGATACCTGCAAAGG	Chr4:8194588	2	+	43	2	0	0	0	0	0.65
8	AGGACGAGGAGCACTACGGAAGG	Chr4:8194415	3	+	61	2	0	0	0	0	0.65
9	TGTCTTAAGCGGCTTGAGAAGTGG	Chr4:8194638	2	+	48	0	0	0	0	0	0.62

AT4G14210.1, isoform 1



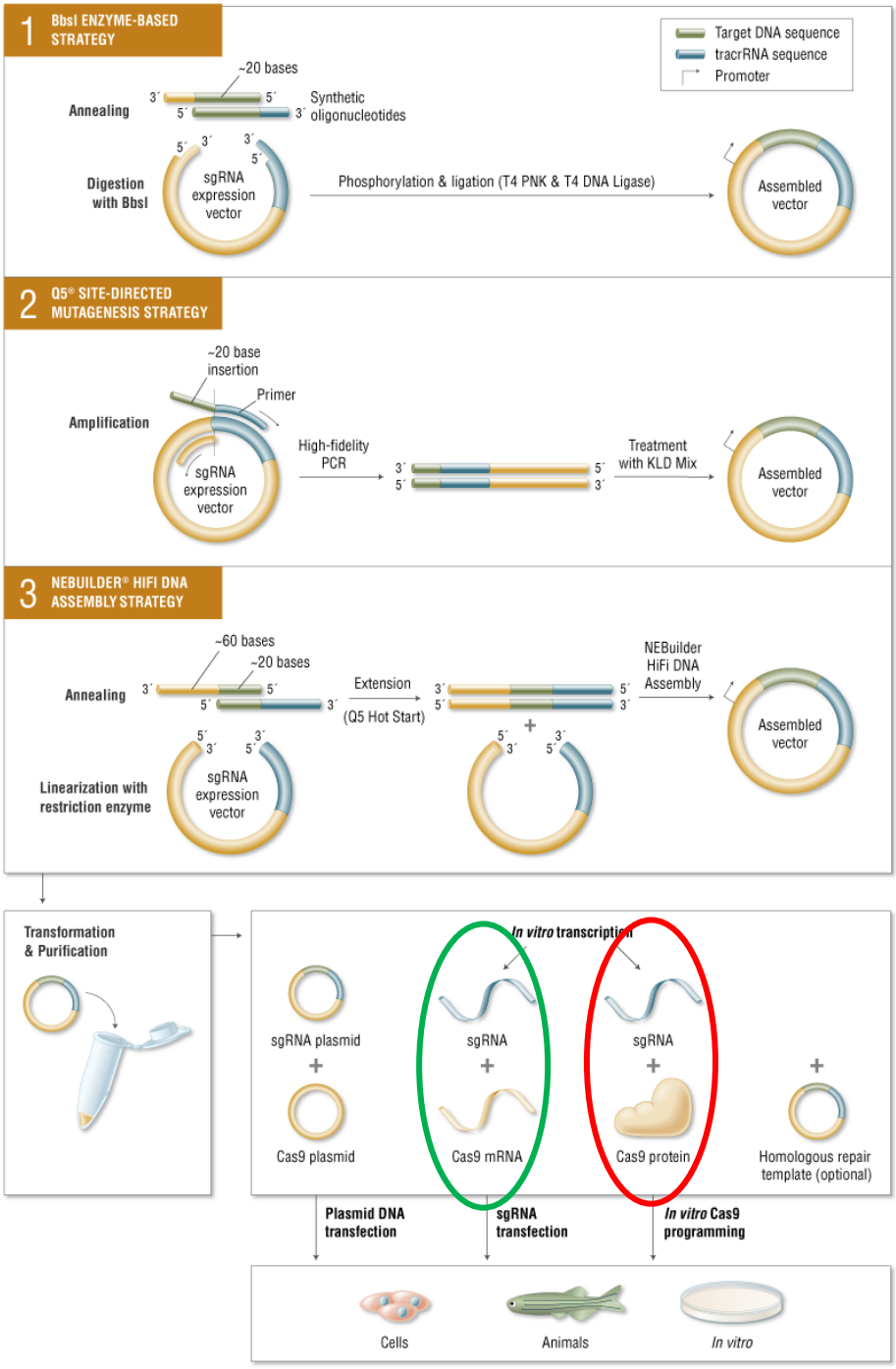
Download an annotated GenBank file of the results here

Download an csv table of the primers here

Gene specific part of sgRNA
CGCTTAAGACAAGAACAAGGAGG
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr4:8194687-8194709	CACTTTCATCTGGAGGTTGTGA	60.1	0	Chr4:8194478-8194500	CTCTAGCTCTGGCCTTGGAATA	60.0	0	0	231
2	Chr4:8194746-8194768	GGTTGTGTTTGGGAATGTTTCT	60.1	0	Chr4:8194478-8194500	CTCTAGCTCTGGCCTTGGAATA	60.0	0	0	290
3	Chr4:8194676-8194698	GGAGGTTGTGAACTAATGGGAC	59.7	0	Chr4:8194478-8194500	CTCTAGCTCTGGCCTTGGAATA	60.0	0	0	220
4	Chr4:8194710-8194732	TTATCAAAACGGGTTTTTGGAG	60.2	0	Chr4:8194478-8194500	CTCTAGCTCTGGCCTTGGAATA	60.0	0	0	254
5	Chr4:8194687-8194709	CACTTTCATCTGGAGGTTGTGA	60.1	0	Chr4:8194471-8194493	CAGTGTTCTCTAGCTCTGGCCT	60.2	0	0	238

3.3 T7 dependent expression of sgRNA and co-transfection with NLS-CAS9 to isolated protoplasts:

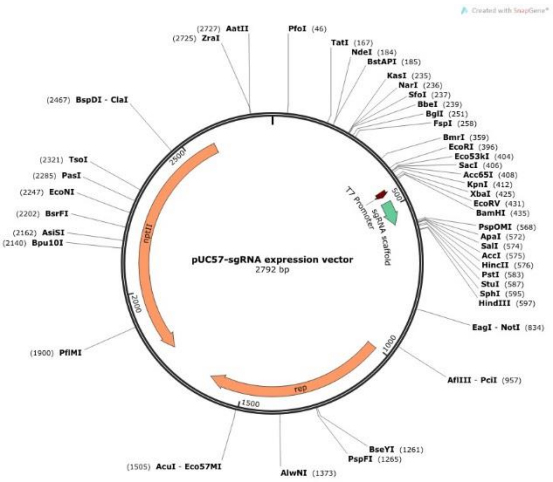


https://www.neb.com/applications/cloning-and-synthetic-biology/genome-editing/sgrna-template-construction-for-cas9-gene-editing

3.3.1 Subcloning of spacers in T7 dependent sgRNA expressor vector:

The oligos of the beforementioned spacers will be ordered after the addition of two adapters at the 5' of each oligo. These oligos will generate overhangs after the annealing of primers that are

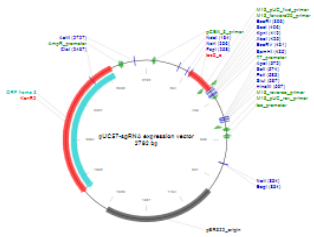
compatible with the two BsaI sites in the vector (see below (Shen et al. 2014)). For the method of spacer production and subcloning see (Shen et al. 2014; Liu et al. 2015).



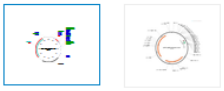
pUC57-sgRNA expression vector

T7 promoter is highlighted in yellow. Two Bsa I restriction sites, shaded in purple, are used for inserting annealed oligos for the target sequence. The scaffold of the sgRNA is underlined. A Dra I restriction site, shaded in blue, is used to linearize the plasmid for *in vitro* transcription.

```
tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggatgccg
ggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggctggcttaactatgcggcatcagagcagattg
tactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggcgccattcgccattca
ggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctggcgaaaggggatgtgctgcaaggc
gattaagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacggccagtgaattcgagctcggtacctcgcgaa
tgcatctagatatcggatcccTAATACGACTCACTATAGGTGAGACCGAGAGAGGGTCTCAGTTTTAGAGCTAGAAATAGCAAG
TTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTAAAgggcccgtcgactgcagaggc
ctgcatgcaagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacga
gccggaagcataaagtgtaaagcctgggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttc
cagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcgccgc
cgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggtt
atccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgtt
gctggcgttttccataggctccgcccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacagg
actataaagatacccaggcgtttcccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtc
cgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaa
gctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaag
acacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaa
gtggtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagt
tggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtttttttgtttgcaagcagcagattacgcgcagaaaaaa
aggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcat
gagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaac
ttggtctgacagttagaaaaactcatcgagcatcaaatgaaactgcaatttattcatatcaggattatcaataccatatttttg
aaaaagccgtttctgtaatgaaggagaaaactcaccgaggcagttccataggatggcaagatcctggtatcggtctgcgattcc
gactcgtccaacatcaatacaacctattaatttcccctcgtcaaaaataaggttatcaagtgagaaatcaccatgagtgacgac
tgaatccggtgagaatggcaaaagttatgcatttctttccagacttgttcaacaggccagccattacgctcgtcatcaaaatc
actcgcatcaaccaaaccgttattcattcgtgattgcgcctgagcgagacgaaatacgcgatcgctgttaaaaggacaattaca
aacaggaatcgaatgcaaccggcgcaggaacactgccagcgcatcaacaatattttcaccctgaatcaggatattcttctaatac
ctggaatgctgtttttccagggatcgcagtggtgagtaaccatgcatcatcaggagtacggataaaatgcttgatggtcggaag
aggcataaatttccgtcagccagtttagtctgaccatctcatctgtaacatcattggcaacgctacctttgccatgtttcagaaa
caactctggcgcatcgggcttcccatacaatcgatagattgtcgcacctgattgcccgacattatcgcgagcccatttatacccc
atataaatcagcatccatgttggaatttaatcgcggcctagagcaagacgtttcccgttgaatatggctcataacttcttcctttt
tcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaataggg
ggttccgcgcacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaataggcg
tatcacgaggccctttcgtc
```



Enlarge | View all sequences



PURPOSE

(Empty Backbone) For in vitro transcription of sgRNA from the T7 promoter.

DEPOSITING LAB

Xingxu Huang

PUBLICATION

Shen et al Nat Methods. 2014 Mar 2. doi: 10.1038/nmeth.2857. (How to cite)

SEQUENCE INFORMATION

Depositor Sequences: Full (1)

Addgene Sequences: Partial (2)

ORDERING

Item	Catalog #	Description	Quantity	Price (USD)	
Plasmid	51132	Plasmid sent as bacteria in agar stab	1	$65	Add to Ca

Available to Academic and Nonprofits Only

BACKBONE

Vector backbone: pUC57
(Search Vector Database)

Vector type: Mammalian Expression, CRISPR

GROWTH IN BACTERIA

Bacterial Resistance(s): Kanamycin

Growth Temperature: 37°C

Growth Strain(s): DH5alpha

Copy number: High Copy

GENE/INSERT

Gene/Insert name: sgRNA scaffold

Promoter: T7

CLONING INFORMATION

Cloning method: Restriction Enzyme

5′ cloning site: BsaI (not destroyed)

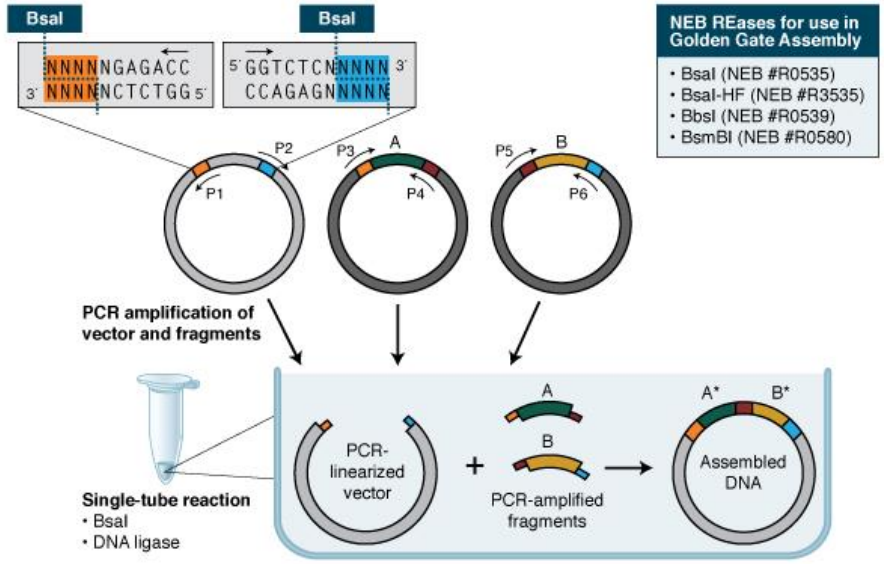
3′ cloning site: BsaI (not destroyed)

5′ sequencing primer: pBAC

3′ sequencing primer: Lac-promoter

DNA constructs. The Cas9 expression construct pST1374-Cas9-N-NLS-Flag-linker (Addgene 44758) was optimized for nuclear import and contains a CMV promoter for expression in mammalian cells and a T7 promoter for *in vitro* transcription of *Cas9* mRNA[11]. This plasmid also contains a blasticidin selection cassette for transient enrichment of transfected cells expressing Cas9. To generate the vectors encoding Cas9(D10A) and Cas9(H840A) nickases, pST1374-Cas9-N-NLS-flag-linker was mutagenized by PCR amplification using PrimeSTAR HS DNA polymerase (Takara, DR010A) and the In-Fusion PCR Cloning kit (Clontech, 638909). The primers used for site-specific mutagenesis are Mut D10A forward (For): 5′-GGACTGGCTATCGGGACAAACTCC GTTGGCTGGGC; Mut D10A reverse (Rev): 5′-CCCGATAGC CAGTCCAATAGAGTATTTCTTGTCTCTAG; Mut H840A For: 5′-CGTGGACGCTATTGTTCCACAGTCCTTCCTCAAAGATG; and Mut H840A Rev: 5′- AACAATAGCGTCCACGTCATAATCG GACAGCCGGTTG. The pUC57-sgRNA expression vector used for *in vitro* transcription of sgRNAs contains a synthetic fragment composed of a T7 promoter, two BsaI sites and the sgRNA cloned into the pUC57 vector (Thermo, SD0171; modified by Taihe, Beijing). pGL3-U6-sgRNA-PGK-Puro vector used for expression of sgRNAs in cells contains the U6-PGK-Puro fragment amplified from pLKO.1 (Addgene 8453) inserted into pGL3-Basic plasmid backbone (Promega, E1751). The sgRNA scaffold was PCR-amplified from pUC57-sgRNA and inserted downstream of *U6* promoter using AgeI and EcoRI. Oligos for the generation of sgRNA expression plasmids (**Supplementary Table 7**) were annealed and cloned into the BsaI sites of pUC57-sgRNA or pGL3-U6-sgRNA-PGK-Puro. Annotated sequences of pST1374-Cas9-N-NLS-flag-linker-D10A (Addgene 51130), pST1374-Cas9-N-NLS-flag-linker-H840A (Addgene 51129), pUC57-sgRNA (Addgene 51132) and pGL3-U6-sgRNA-PGK-Puro (Addgene 51133) plasmids are presented in the **Supplementary Note**.

***In vitro* transcription.** Cas9 expression plasmid was linearized with AgeI and *in vitro* transcribed using the T7 Ultra Kit (Ambion, AM1345). mRNA was purified by RNeasy Mini Kit (Qiagen, 74104). pUC57-sgRNA expression vectors were linearized by DraI and *in vitro*–transcribed using the MEGAshortscript Kit (Ambion, AM1354). sgRNAs were purified by MEGAclear Kit (Ambion, AM1908) and recovered by alcohol precipitation.



* While A and B insert sequences involved in 4-base overlaps are shown in separate colors for clarity, the actual assembly is seamless; 4-base overlaps are insert derived.

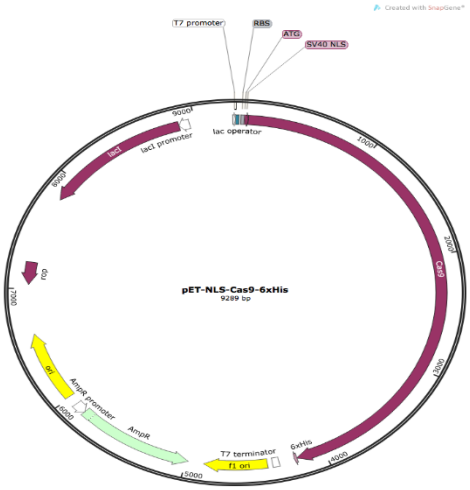
https://www.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/golden-gate-assembly

3.3.2 CAS9 sources:

3.3.2.1 Protein

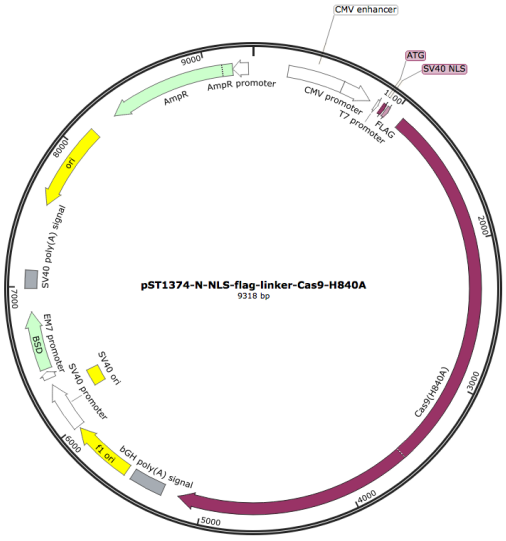
1- Protein production, cleavage, and purification of CAS9 from PMJ806 (no. 39312). Also, we will order the plasmid that contains NLS-CAS9 (PET-NLS-Cas9-6xHis no. 62934). The second plasmid was modified from the first, but showed to be more efficiently working in vivo, also it does not need cleavage of MBP as in the first vector. In case if we will work for the second, we will only use His tag for purification etc (find a good protocol). Also for suggestions of amount of gRNAs and proteins we will look at (Zuris et al. 2015), specially supplemental figure 6 and (Woo et al. 2015).

2- Buying ready made NLS-CAS9 for example from NEB (https://www.neb.com/products/m0641-cas9-nuclease-nls-s-pyogenes).



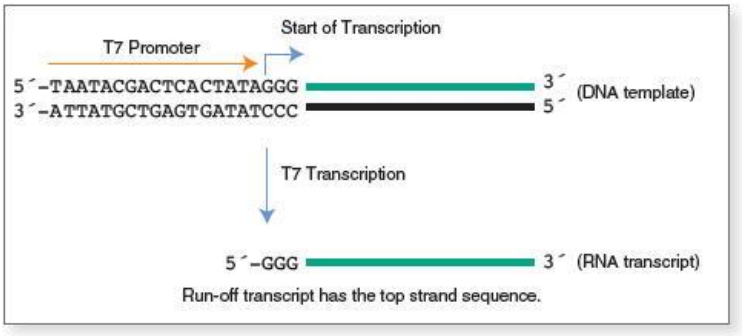
3.3.2.2 Transcripts

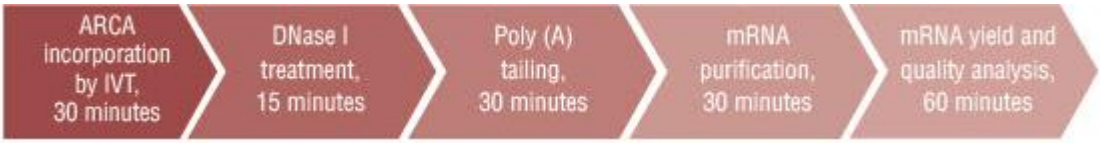
In vitro transcription of NLS-CAS9 from the plasmid pST1374-NLS-flag-linker-Cas9 (no. 44758, (Shen et al. 2014)).



3.3.3 In vitro transcription of sgRNA and NLS-CAS9

E2050S (https://www.neb.com/products/e2050-hiscribe-t7-quick-high-yield-rna-synthesis-kit) and E2060S (https://www.neb.com/products/e2060-hiscribe-t7-arca-mrna-kit-with-tailing), will be used for the transcription of sgRNAs and NLS-CAS9, respectively.



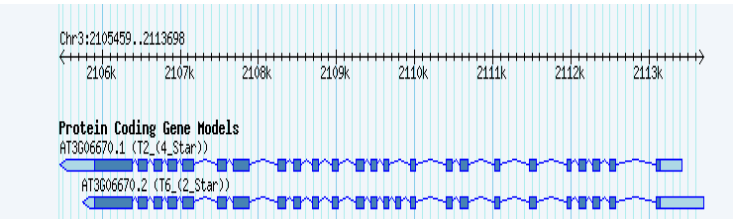


Purifying methods and kits will be chosen and ordered?

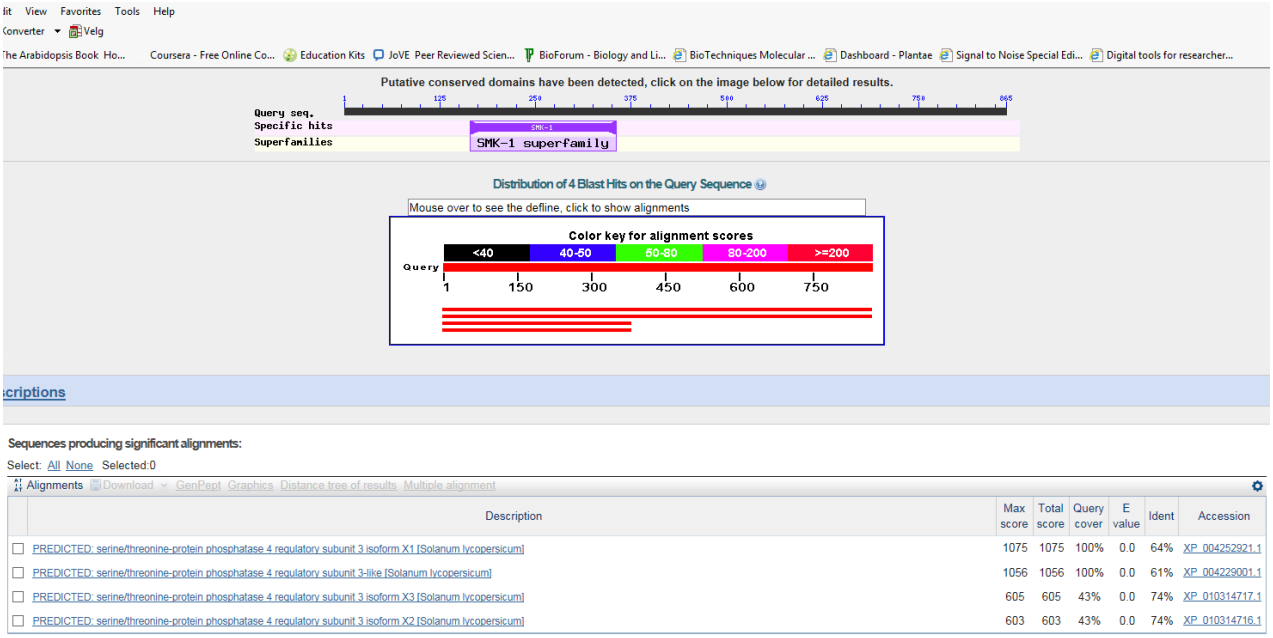
3.4 Finding PSY2L homologs in Tomato

Arabidopsis PSY2L AT3G06670.1

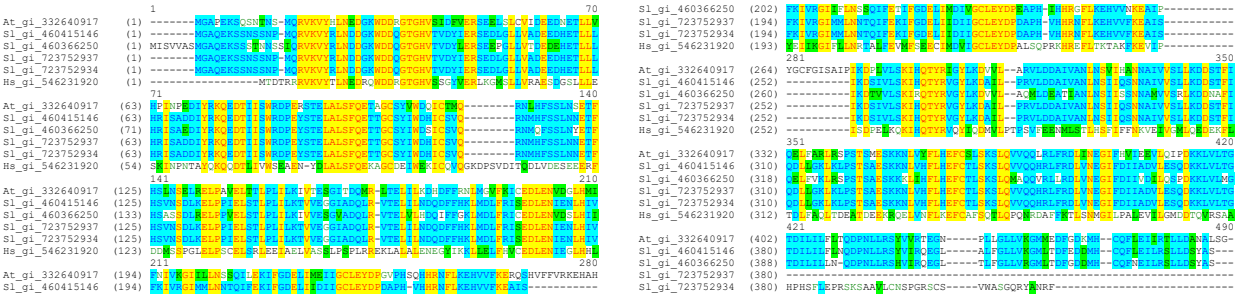
```
  1 MGAPEKSQSN TNSMQRVKVY HLNEDGKWDD RGTGHVSIDF VERSEELSLC
 51 VIDEEDNETL LVHPINPEDI YRKQEDTIIS WRDPERSTEL ALSFQETAGC
101 SYVWDQICTM QRNLHFSSLN SETFHSLNSE LRELPAVELT TLPLILKIVT
151 ESGITDQMRL TELILKDHDF FRNLMGVFKI CEDLENVDGL HMIFNIVKGI
201 ILLNSSQILE KIFGDELIME IIGCLEYDPG VPHSQHHRNF LKEHVVFKEA
251 IPIKDPLVLS KIHQTYRIGY LKDVVLARVL DDAIVANLNS VIHANNAIVV
301 SLLKDDSTFI QELFARLRSP STSMESKKNL VYFLHEFCSL SKSLQVVQQL
351 RLFRDLINEG IFHVIEEVLQ IPDKKLVLTG TDILILFLTQ DPNLLRSYVV
401 RTEGNPLLGL LVKGMMEDFG DKMHCQFLEI IRTLLDANAL SGGAQRANIM
451 DIFYEKHLPE LVDVITASCP EKSSNASEGA ARRIFTKPEV LLNICELLCF
501 CIMQDASRTK CSFLQNNVTE KVLHLTRRKE KYLVVAAIRF VRTLLSVHDD
551 YVQNYVVKNN LLKPIIDVFI ANGTRYNLLN SAVLDLLEHI RKGNATLLLK
601 YIVDTFWDQL APFQCLTSIQ AFKVKYEQCL ESAGPKSTSD AVDPRRRVDE
651 RALEKEEEDY FNEDSDEEDS ASASNTQKEK PASNIQKEQP KPHLSNGVAA
701 SPTSSSPRSG GLVDYEDDED DEDYKPPPRK QPEASEDEEG ELLRLKRKSA
751 LVEREQEPSK KPRLGKSSKR ENVFAVLCST LSHAVLTGKK SPGPAGSAAR
801 SIVAKGAEDS KSSEENNSSS SDDENHKDDG VSSSEHETSD NGKLNGEESL
851 VVAPKSSPEM AVNGS
```

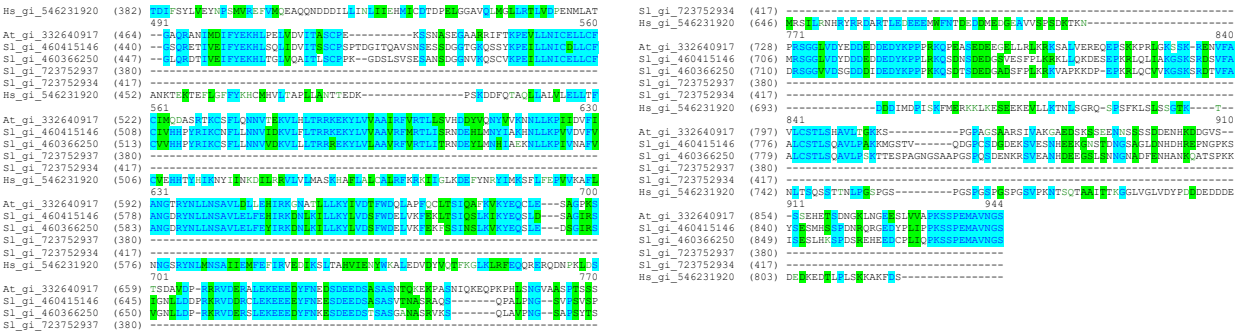


3.4.1 Alignment of PSY2L towards Tomato Solanum lycopersicum (taxid:4081) in NCBI:

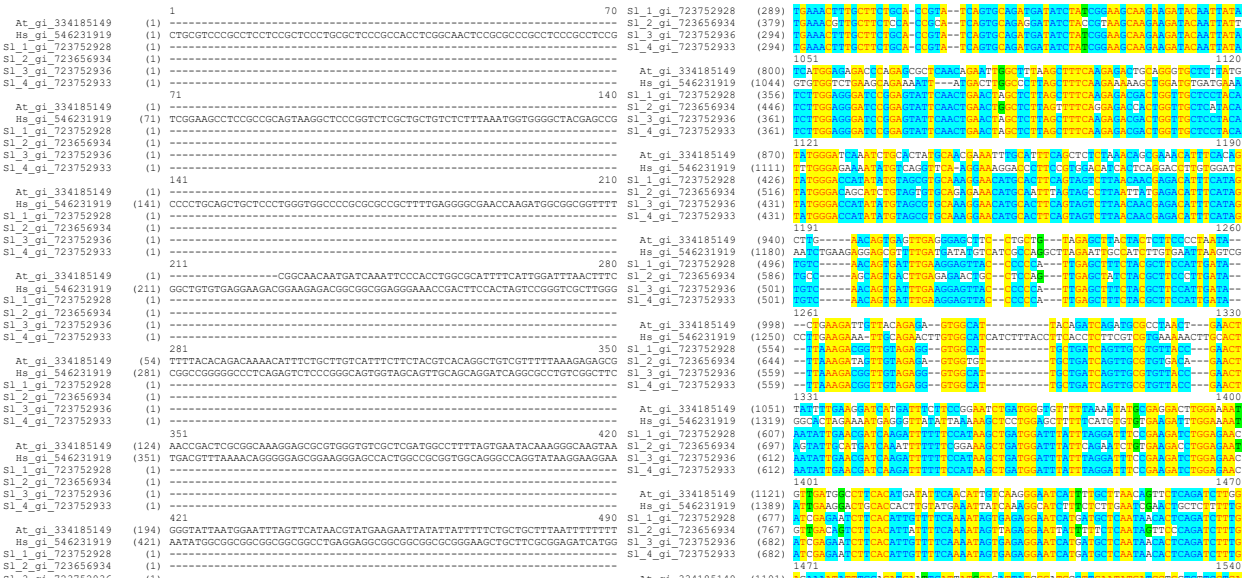


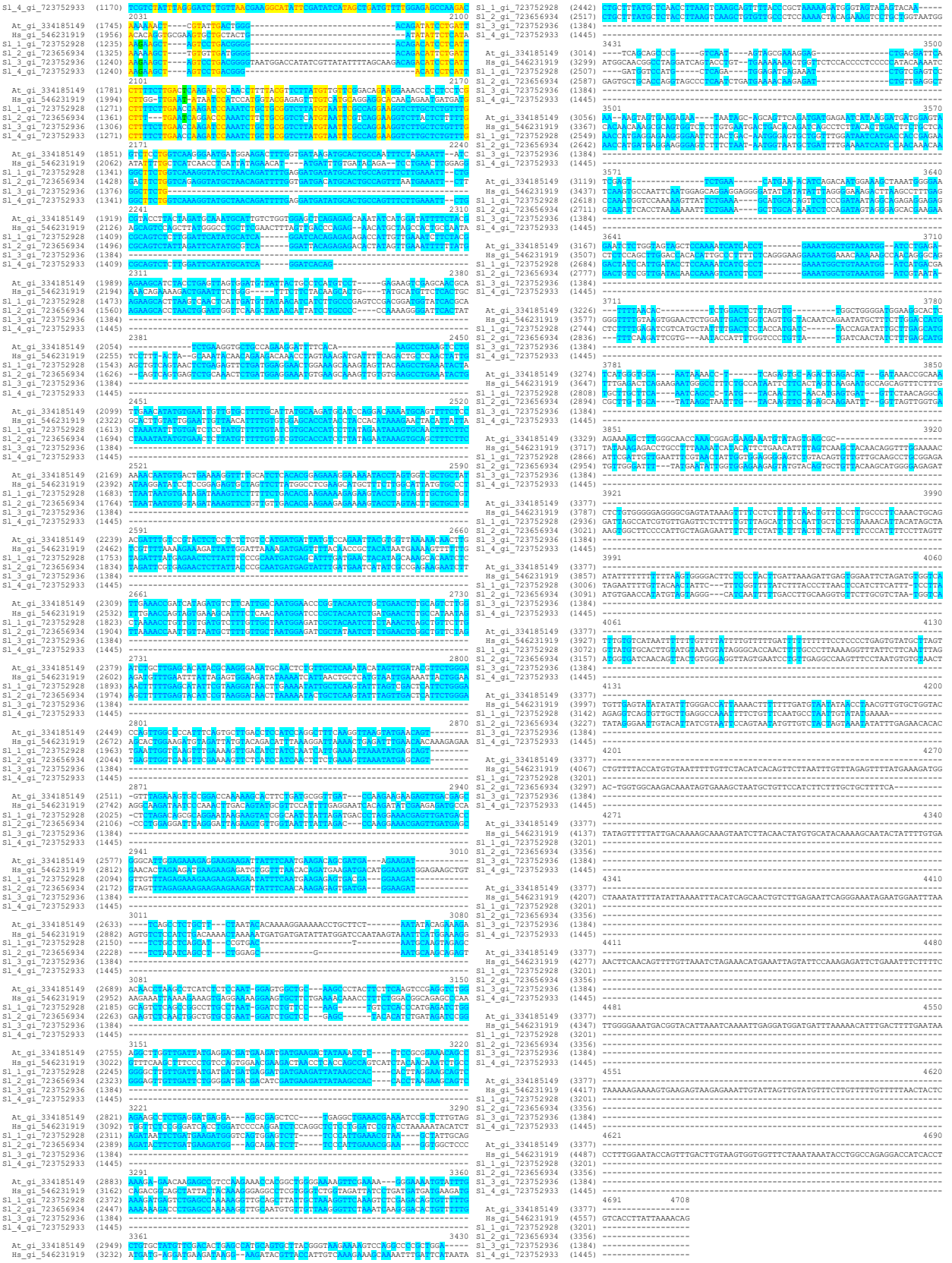
3.4.2 Alignment of PSY2L from Arabidopsis, human, and tomato 4 homologs appeared in the above alignment:





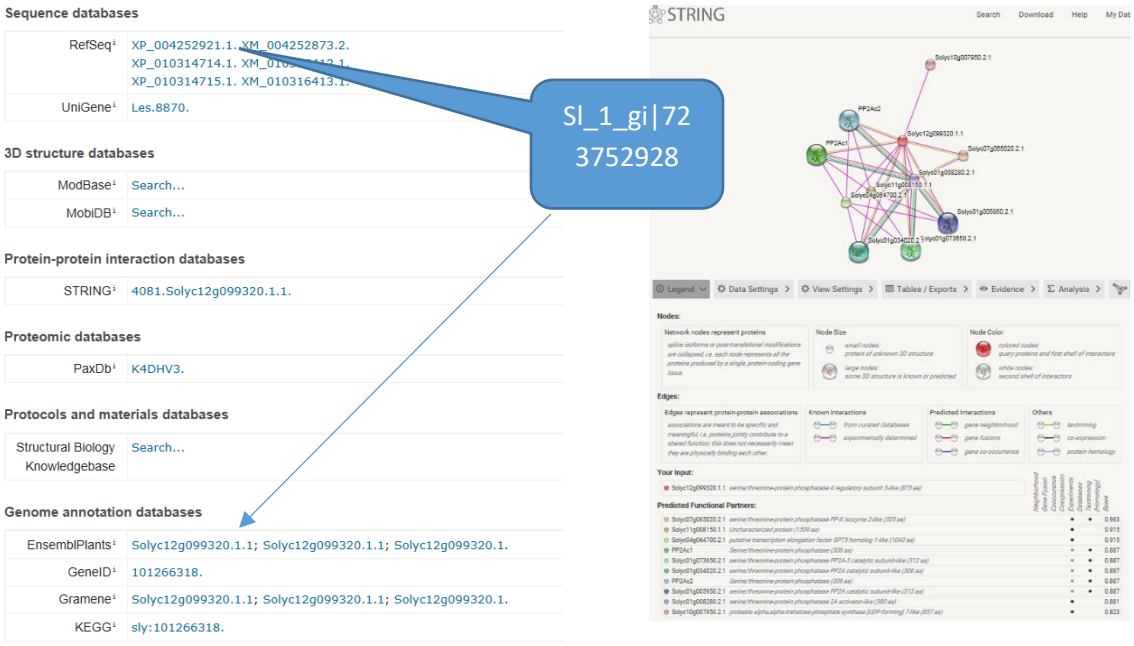
3.4.3 Alignment of PSY2L mRNA from Arabidopsis, human, and tomato 4 homologs appeared in the above alignment:

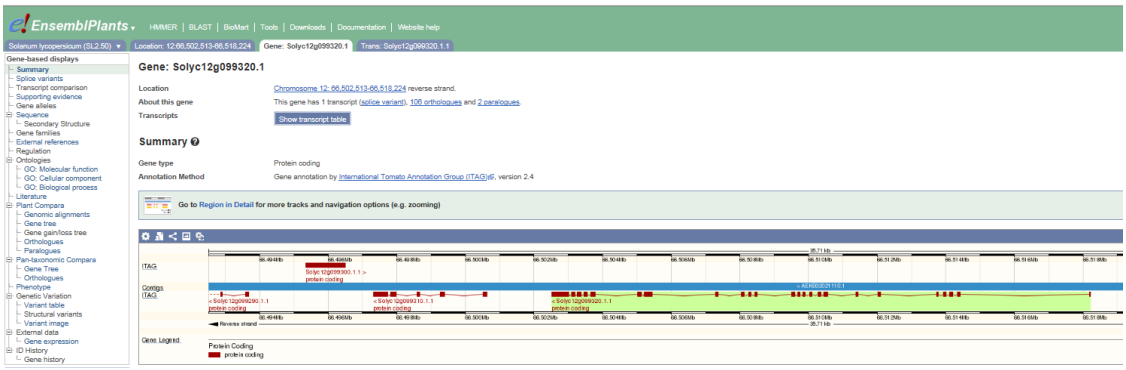




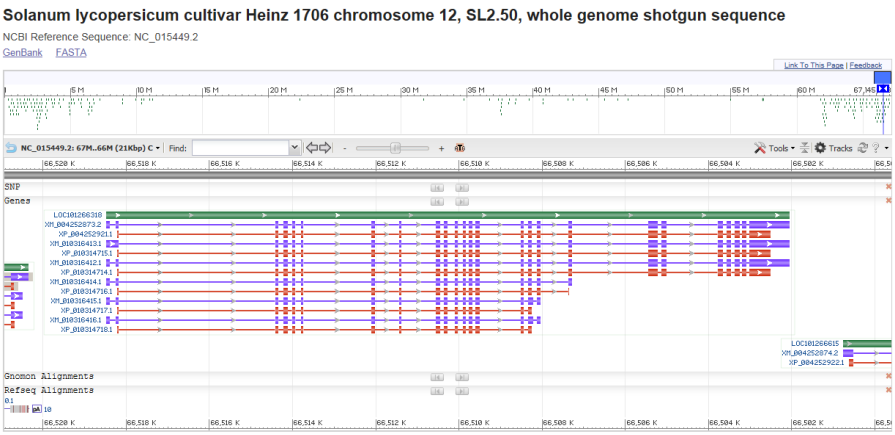
3.4.4 Selecting PSY2L homolog for generating gRNAs

3.4.4.1 Sl-PSY2L-1 (Solyc12g099320.1.1) has 64% homology with Arabidopsis



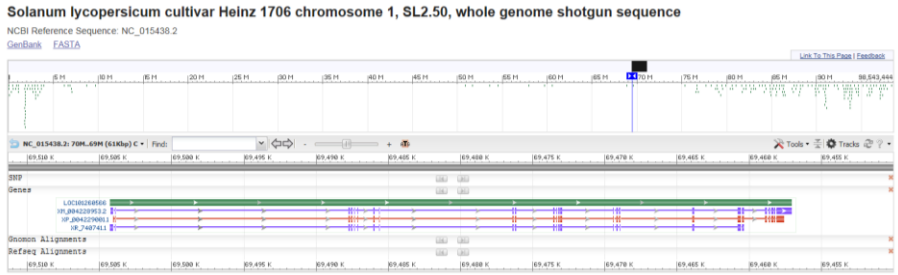


http://www.ncbi.nlm.nih.gov/gene/?term=XP_004252921.1



3.4.4.2 Sl-PSY2L-2 (Solyc01g???) has 61% homology with Arabidopsis

http://www.ncbi.nlm.nih.gov/gene/?term=XP_004229001.1 (61%)…. I could not find any info about its code in any Tomato databases… By searching for gRNAs from its mRNA (from NCBI), I always have hits against the homolog in Chr 1…. However, they are different in size but they are similar in the sequence… Probably they are variants….



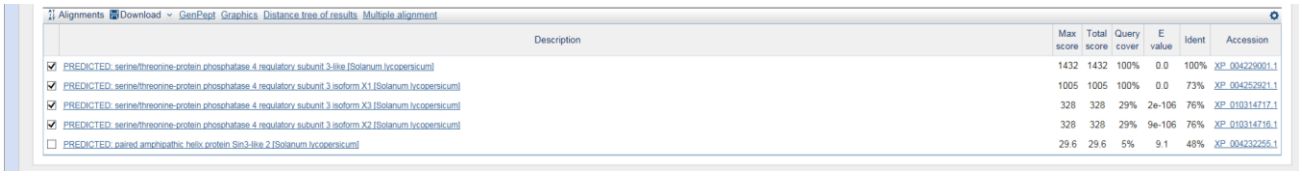
Based on the link ftp://ftp.solgenomics.net/genomes/Solanum_lycopersicum/id_conversion/ and ftp://ftp.solgenomics.net/genomes/Solanum_lycopersicum/id_conversion/tom2_to_solyc_annotated_id40.txt : I searched the annotation of PSY2L and found these two locuses:

SGN-U221820 Solyc12g099320.1.1 100.00 70 0 0 1 70 2386 2455 1e-30 130 Serine/threonine-protein phosphatase 4 regulatory subunit 3 IPR006887, Protein of unknown function DUF625 GO:0005488

SGN-U213697 Solyc01g060080.2.1 100.00 70 0 0 1 70 1043 1112 1e-30 130
Serine/threonine-protein phosphatase 4 regulatory subunit 3 IPR006887, Protein of unknown function DUF625
GO:0005515..... http://www.uniprot.org/uniprot/K4AWB2

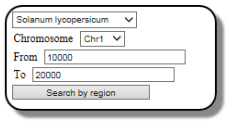
This is from Chromosome one and alignment with the is protein from `Solyc12g099320.1.1` show 58.8% identity: and when aligned against tomato on NCBI taxid 4081, only shows the three variants of `Solyc12g099320.1.1`…. see the alignment window..

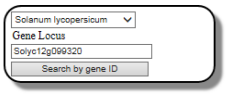
```
                    1                                                          70
Sl_gi_460415146   (1) MGAQEKSSNSSNPMQRVKVYRLNDDGKWDDQGTGHVTVDYIERSEDLGLLVADEEDHETLLLHRISADDI
       _clipseq   (1) ------------------------------------------------------------------------
                   71                                                         140
Sl_gi_460415146  (71) YRKQEDTIISWRDPEYSTELALSFQETTGCSYIWDHICSVQRNMHFSSLNNETFHSVNSDLKELPPIELS
       _clipseq   (1) ------------------------------------------------------------------------
                  141                                                         210
Sl_gi_460415146 (141) TLPLILKTVVEGGIADQLRVTELILNDQDFFHKLMDLFRISEDLENIFNLHIVFKIVRGIMMLNNTQIFE
       _clipseq   (1) ----------------------------------MDLFRICEDLENVLSLHIIFKIVRGILFLNSSQIFE
                  211                                                         280
Sl_gi_460415146 (211) KIFGDELIIDILGCLEYDPGAPHVHHFNFLKEHVVFKEAISIKDSIVLSKIHQTYRVGYLKDALLPRVLD
       _clipseq  (37) TIFGDELIMDIVGCLEYDPGAPHIHHRGFLKEHVVNKEAIPIKDTVVLSKIRQTYRVGYLKDVVLAQMLD
                  281                                                         350
Sl_gi_460415146 (281) QAIVANLNSIIQSNNATVVSLLKDDSTFIQDLLGKLKLPSTSAESKKNLLHFLHEFCTLSKSLQVVQQHR
       _clipseq (107) EATIANLNSIISSNNAMVVSRLKDDNAFIQELFVKLRSPSTSAESKKKLLHFLHEFCTLSKSLQMAQQVR
                  351                                                         420
Sl_gi_460415146 (351) LFRDLVNEGIFDIIADVLESQDKKLVLTGTDILILFLNQDPNLLRSMVIRQEGLALFGLLVKGMLTDFED
       _clipseq (177) LLRDLVNEGIFDIIVDILQSPDKKLVLMGTDILLL-LNQDPNLLRSHVIRQEGLTLFGLLVKGMLTDFGD
                  421                                                         490
Sl_gi_460415146 (421) DMHCQFLEILRSLLDSYASGSQRETIVEIFYEKHLSQLIDVITSSCPSPTDGITQAVSNSESSDGGTGKQ
       _clipseq (246) DMHCQFNEILRSLLDSYASGLQRDTIVEIFYEKHLIGLVQAITLSCPPKGDSLS--VSESANSDGGNVKQ
                  491                                                         560
Sl_gi_460415146 (491) SSYKPEILLNICDLLCFCTVHHPYRIKCNFLLNNVLDKVLFLTRRKEKYLVVAAVRFMRTLISRNDEHLM
       _clipseq (314) SCVKPEILLNICELLCFCVVHHPYRIKCSFLLNNVMDKVLLLTRRREKYLVLAAVRFVRTLITRNDEYLM
                  561                                                         630
Sl_gi_460415146 (561) NVIAKHNLLKFVVDVFVANGDRYNLLNSAVLELFEHIRKDNLKILLKYLVDSFWDELVKFEKLTSIQSLK
       _clipseq (384) NHIAEKNLLKFIVNAFVANGDRYNLLNSAVLELFEYIRKDNLKILLKYLVDSFWDELVKFEKFFSSINSLK
                  631                                                         700
Sl_gi_460415146 (631) IKYEQSLDSAGIRSIGNLLDDPRKRVLDRCLEKEEEEYFNEESDEEDSASASVTNASRAQSCPALPNGSV
       _clipseq (454) VKYEQSLDDSGIRSVGNLLD-PRKRVLERSLEKEEEDYFNKESDEEDSTSASGANASRVKSQLAVPNGSA
                  701                                                         770
Sl_gi_460415146 (701) PSVSPMRSGGLVDYDDDEDDEDYKPPLRKQSDNSDEDGSVESFPLKRFLLQKDRSEPKRLQLLAKGSKSR
       _clipseq (523) PSYISDRSGGMVDSGDDEDIDEDYKPPPKKQSDTSDEDGA-DSFPLKRFVAPKKDPEPKRLQCVVKGSKSR
                  771                                                         840
Sl_gi_460415146 (771) DSVFAALCSTLSQAVLPKKM------GSTVQDGPCSDGDEKSVESNHEEKGNSTDNGSAGLGNHDHREP
       _clipseq (592) DTVFAALCSTLSQAVLPSKTTESPAGNGSAAPGSPQSDENKKSVESNHDEEGSLSNNGNADFENHANKQA
                  841                 879
Sl_gi_460415146 (835) NGPKSYSESMHSSPDNPQRGEDYPLIPPKSSPEMAVNGS
       _clipseq (662) TSPKKISESLHKSPDSKEHEEDCPLIQPKSSPEMAVNGS
```



3.4.5 Identifying predicted spacers for the selected Sl-PSY2L

3.4.5.1 Using CRISPR plant and other websites for prediction of the gene Sl-PSY2L-1





Your query is: Solyc12g099320 in Chr12 from 64843563 to 64859274

Class0.0 gRNA

SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr12:64845882-64845902:c	NA	NA	TATAGCGCGGTATACGTGGT	AGGCGCATGT	-	intron
Chr12:64845886-64845906:c	NA	4	GAAGTATAGCGCGGTATACG	TGGTAGGCGC	-	intron
Chr12:64847946-64847966	NA	4	GTACGCCCACACGTGCACTA	GGGAACAAGC	+	intron
Chr12:64855441-64855461:c	NA	5	GTCTATCGCCTGAATGACGA	TGGAAAATGG	-	exon

Total of 4 class0.0 gRNA seeds were found in this region

Class1.0 gRNA

SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr12:64843581-64843601	4	3	ATTTCAGGCGATGATTTTGG	AGGTATCAAT	+	exon
Chr12:64843634-64843654:c	4	4	TGCACAGTTCTCCCGATAAT	AGGCAGAGAG	-	exon
Chr12:64843680-64843700:c	3	4	CATGACCACCGAGAACCAAA	TGGTCCAAAA	-	exon
Chr12:64843672-64843692	3	3	TTGGACCATTTGGTTCTCGG	TGGTCATGAT	+	exon
Chr12:64843708-64843728:c	4	3	TGACAATGGGAGTGCTGGTT	TGGATAATCA	-	exon
Chr12:64843713-64843733:c	4	3	TCTACTGACAATGGGAGTGC	TGGTTTGGAT	-	exon
Chr12:64843722-64843742:c	3	3	AAGGGGAATTCTACTGACAA	TGGGAGTGCT	-	exon
Chr12:64843739-64843759:c	4	3	AGTCCAACCATGAGGAGAAG	GGGAATTCTA	-	exon
Chr12:64843740-64843760:c	3	4	GAGTCCAACCATGAGGAGAA	GGGGAATTCT	-	exon
Chr12:64843741-64843761:c	4	4	CGAGTCCAACCATGAGGAGA	AGGGGAATTC	-	exon
Chr12:64843733-						

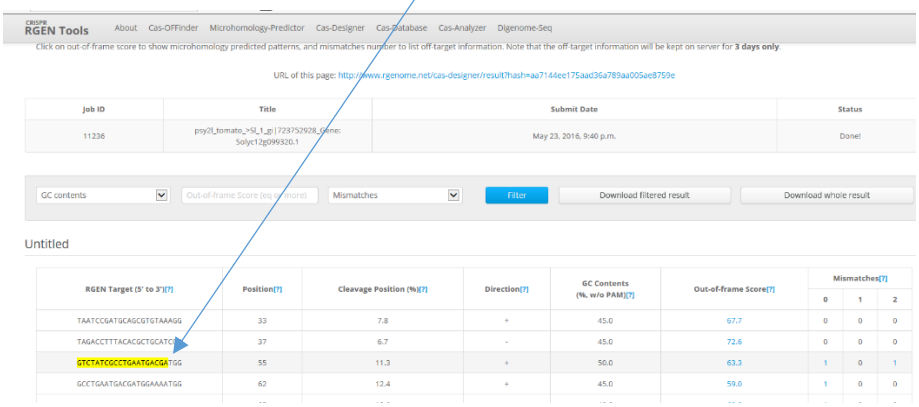
Class0.0 gRNA						
SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr12:64855441-64855461:c	NA	5	GTCTATCGCCTGAATGACGA	TGGAAAATGG	-	exon

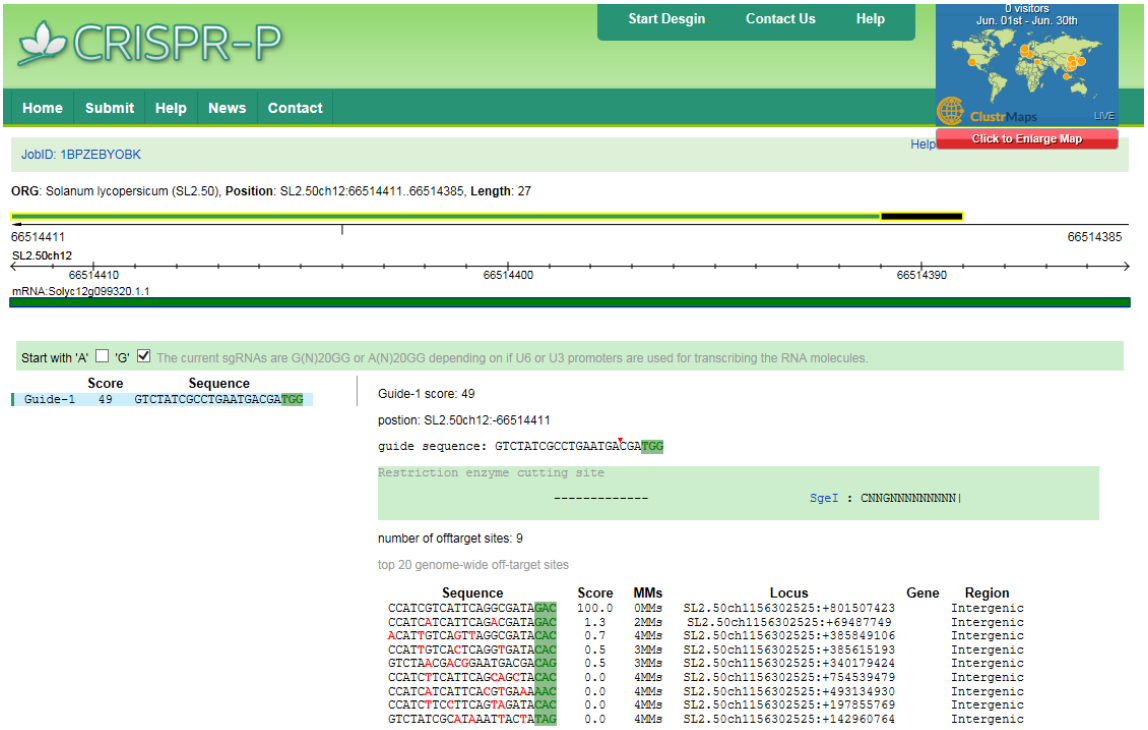
gRNA (Spacer was shown in upper-case):
5'-GTCTATCGCCTGAATGACGAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GTCTATCGCCTGAATGACGA-3'
5'-TCGTCATTCAGGCGATAGAC-3'

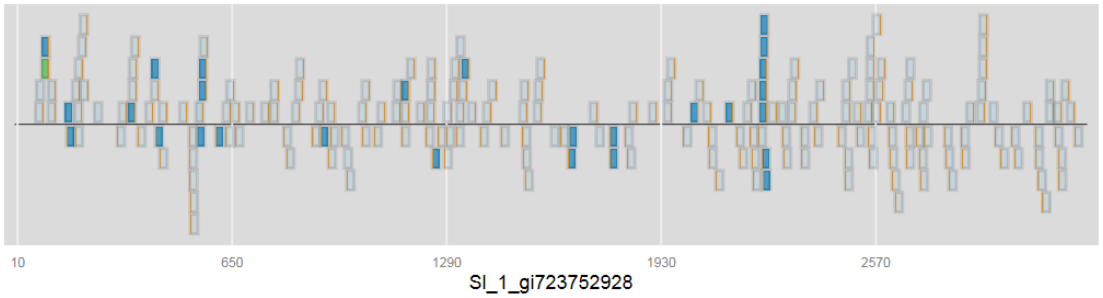
GC content of Spacer sequence: 0.5
Potential Pol III terminator (TTTTT): null
0 from 149 REs recoginze Cas9 cut region (+7 to -13bp):

This candidate spacer was also recommended from other prediction website:





And the CCTOP website http://crispr.cos.uni-heidelberg.de/cgi-bin/search.py?sid=e11103b8da273ef64a2808b3c24810b9ccc89ff2 , when mRNA was used



Legend for off-target site positon: E = exonic; I = intronic; - = intergenic

							Interpro domain(s) IPR006887 Protein of unknown function DUF625

T74 out of 220

<Previous Next>

Sequence: GTCTATCGCCTGAATGACGATGG

Oligo pair fwd: ATTGGTCTATCGCCTGAATGACGA rev: AAACTCGTCATTCAGGCGATAGAC

Coordinates	strand	MM	target_seq	PAM	distance		gene name	gene id
SL2.50ch12:66514389-66514411	-	0	GTCTATCG [CCTGAATGACGA]	TGG	0	E	Serine/threonine-protein phosphatase 4 regulatory subunit 3 (AHRD V1 **-- PP4R3_DANRE); contains Interpro domain(s) IPR006887 Protein of unknown function DUF625	Solyc12g099320.1.1

The following is found also in another research engine as number 1 …. Look at 3.4.5.2

Chr12:64853090-64853110	4	4	AAGCGTAGAAAGCTCAATGG	GGGGTAACTC	+	exon

gRNA (Spacer was shown in upper-case):
5'-AAGCGTAGAAAGCTCAATGGgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-AAGCGTAGAAAGCTCAATGG-3'
5'-CCATTGAGCTTTCTACGCTT-3'

GC content of Spacer sequence: 0.45
Potential Pol III terminator (TTTTT): null
0 from 149 REs recoginze Cas9 cut region (+7 to -13bp):

SI_1_gi723752928

Legend for off-target site positon: E = exonic; I = intronic; - = intergenic

T211 out of 220

<Previous Next>

Sequence: AAGCGTAGAAAGCTCAATGGGGG

Oligo pair fwd: ATTGAAGCGTAGAAAGCTCAATGG rev: AAACCCATTGAGCTTTCTACGCTT

Coordinates	strand	MM	target_seq	PAM	distance		gene name	gene id
SL2.50ch12:66512041-66512063	+	0	AAGCGTAG [AAAGCTCAATGG]	GGG	0	E	Serine/threonine-protein phosphatase 4 regulatory subunit 3 (AHRD V1 **-- PP4R3_DANRE); contains Interpro domain(s) IPR006887 Protein of unknown function DUF625	Solyc12g099320.1.1

This following is found also in another research engine as number 2 …. Look at 3.4.5.2

Chr12:64849511-64849531:c	3	3	TTTGAGTAAGAGTTTGCAGG	TGGTCCAGCA	-	exon

gRNA (Spacer was shown in upper-case):
5'-TTTGAGTAAGAGTTTGCAGGgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-TTTGAGTAAGAGTTTGCAGG-3'
5'-CCTGCAAACTCTTACTCAAA-3'

GC content of Spacer sequence: 0.4
Potential Pol III terminator (TTTTT): null
1 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
HpyCH4V cut TGCA

3.4.5.2 Using WustL for prediction of mRNA copy

Primers for gRNA: >Sl_1_gi|723752928 was copied into the website http://crispr.wustl.edu for giving potential places for gRNA targeting:

gRNA and Target Gene Description:

gRNA Sequence	5'-aagcgtagaaagctcaatgg-3' (20 n.t.)
Potency Score	81
gRNA Location	525
Target Strand	antisense
Target Sequence Length	1637

Coding Sequence

```
   1 cagggcacgaa cacttcagaa gaaaaagaaa cgaagagaga gaaagaagag agagaaagaa
  61 gagaagattc actactcatc aatggcggca gtgtgaaatt tgggttgtag aagtttctat
 121 gggtgctcaa gagaagtcat caaactctag taatccgatg cagcgtgtaa aggtctatcg
 181 cctgaatgac gatggaaaat gggatgatca aggaacgggt catgttactg tagattatat
 241 agagagatca gaagatctag gattgcttgt agctgatgaa gaagatcatg aaacttttgct
 301 tctgcaccgt atcagtgcag atgatatcta tcggaagcaa gaagatacaa ttatatcttg
 361 gagggatccg gagtattcaa ctgaactagc tcttagcttt caagagacga ctggttgctc
 421 ctacatatgg gaccatatat gtagcgtgca aaggaacatg cacttcagta gtcttaacaa
 481 cgagacattt catagtgtca acagtgattt gaaggagtta ccccccattg agctttctac
 541 gcttccattg atatttaaaga cggttgtaga gggtggcatt gctgatcagt tgcgtgttac
 601 cgaactaata ttgaacgatc aagatttttt ccataagctg atggatttat ttaggatttc
 661 cgaagatctg gagaacatcg agaatcttca catttgttttc aaaatagtga gaggaatcat
 721 gatgctcaat aacactcaga tctttgaaaa aatatttggt gatgagctga taatagatat
 781 tattgggtgt cttgaatatg atccagatgc tcctcatgtt catcatcgca atttctcaa
 841 agagcatgtg gtctttaagg aggctatatc tcgatagtcc tatcgaagat
 901 acatcaaaca taccgagttg gctatctgaa ggatgccatt ttgcctagag tgttggatga
 961 tgcaatagtt gcaaaccctta attccataat ccagtcaaac aatgcaattg ttgtatctct
1021 cttaaaagat gatagcacct tcattcagga tttgcttgga aagttgaagt tgccttctac
1081 atctgcagaa tcaaagaaaa atttggtgca ctttttgcac gagttttgta ctttgagtaa
1141 gagtttgcag gtggtccagc aacatcgtct atttagggat cttgttaacg aaggcatatt
1201 cgatatcata gctgatgttt tggagagcca agacaagaag ctagtcctga cggggacaga
1261 catcctcatt cttttcttga accaagatcc aaatctgctg cggtcttatg taattcgcca
1321 ggaaggtctt gctctgtttg ggcttctggt caaaggtatg ctaacagatt ttgaggatga
1381 tatgcactgc cagtttcttg aaatttctgcg cagtctcttg gattccatatg catcaggatc
1441 acagagagag accattgttg aaatcttcta cgagaagcac ttaagtcaac tcattgatgt
1501 tataacatca tcttgcccga gtccgacgga tggtatcacg caagctgtca gtaactctga
1561 gagttctgat ggaggaactg gaaagcaaag tagttacaag cctgaaatac tactaaatat
1621 ttgtgatctc ctatgtt
```

gRNA and Target Gene Description:

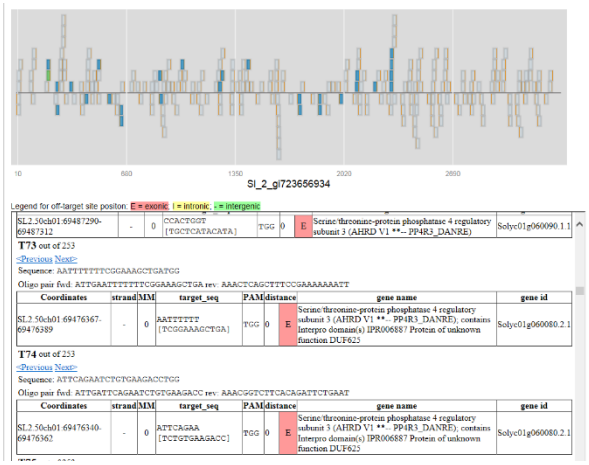
gRNA Sequence	5'-tttgagtaagagtttgcagg-3' (20 n.t.)
Potency Score	80
gRNA Location	1132
Target Strand	sense
Target Sequence Length	1637

Coding Sequence

```
   1 cagggcacgaa cacttcagaa gaaaaagaaa cgaagagaga gaaagaagag agagaaagaa
  61 gagaagattc actactcatc aatggcggca gtgtgaaatt tgggttgtag aagtttctat
 121 gggtgctcaa gagaagtcat caaactctag taatccgatg cagcgtgtaa aggtctatcg
 181 cctgaatgac gatggaaaat gggatgatca aggaacgggt catgttactg tagattatat
 241 agagagatca gaagatctag gattgcttgt agctgatgaa gaagatcatg aaacttttgct
 301 tctgcaccgt atcagtgcag atgatatcta tcggaagcaa gaagatacaa ttatatcttg
 361 gagggatccg gagtattcaa ctgaactagc tcttagcttt caagagacga ctggttgctc
 421 ctacatatgg gaccatatat gtagcgtgca aaggaacatg cacttcagta gtcttaacaa
 481 cgagacattt catagtgtca acagtgattt gaaggagtta ccccccattg agctttctac
 541 gcttccattg atatttaaaga cggttgtaga gggtggcatt gctgatcagt tgcgtgttac
 601 cgaactaata ttgaacgatc aagatttttt ccataagctg atggatttat ttaggatttc
 661 cgaagatctg gagaacatcg agaatcttca catttgttttc aaaatagtga gaggaatcat
 721 gatgctcaat aacactcaga tctttgaaaa aatatttggt gatgagctga taatagatat
 781 tattgggtgt cttgaatatg atccagatgc tcctcatgtt catcatcgca atttctcaa
 841 agagcatgtg gtctttaagg aggctatatc tcgatagtcc tatcgaagat
 901 acatcaaaca taccgagttg gctatctgaa ggatgccatt ttgcctagag tgttggatga
 961 tgcaatagtt gcaaaccctta attccataat ccagtcaaac aatgcaattg ttgtatctct
1021 cttaaaagat gatagcacct tcattcagga tttgcttgga aagttgaagt tgccttctac
1081 atctgcagaa tcaaagaaaa atttggtgca ctttttgcac gagttttgta ctttgagtaa
1141 gagtttgcag gtggtccagc aacatcgtct atttagggat cttgttaacg aaggcatatt
1201 cgatatcata gctgatgttt tggagagcca agacaagaag ctagtcctga cggggacaga
1261 catcctcatt cttttcttga accaagatcc aaatctgctg cggtcttatg taattcgcca
1321 ggaaggtctt gctctgtttg ggcttctggt caaaggtatg ctaacagatt ttgaggatga
1381 tatgcactgc cagtttcttg aaatttctgcg cagtctcttg gattccatatg catcaggatc
1441 acagagagag accattgttg aaatcttcta cgagaagcac ttaagtcaac tcattgatgt
1501 tataacatca tcttgcccga gtccgacgga tggtatcacg caagctgtca gtaactctga
1561 gagttctgat ggaggaactg gaaagcaaag tagttacaag cctgaaatac tactaaatat
1621 ttgtgatctc ctatgtt
```

3.4.5.3 Using CCTOP based on mRNA sequence for Sl-PSY2L-2

The CCTOP website http://crispr.cos.uni-heidelberg.de/cgi-bin/search.py?sid=e11103b8da273ef64a2808b3c24810b9ccc89ff2 , when mRNA was used



T73 out of 253

It has 8 off-targets but with 4 mismatches

Sequence: AATTTTTTTCGGAAAGCTGATGG

Oligo pair fwd: ATTGAATTTTTTTCGGAAAGCTGA rev: AAACTCAGCTTTCCGAAAAAAATT

T74 out of 253

It has 4 off-targets but with 4 mismatches

Sequence: ATTCAGAATCTGTGAAGACCTGG

Oligo pair fwd: ATTGATTCAGAATCTGTGAAGACC rev: AAACGGTCTTCACAGATTCTGAAT

3.4.5.4 Using CCTOP based on mRNA sequences for Sl-PSY2L-1 and 2, and identifying possible dual targets

T218 out of 220 (has 6 off-targets more than 4 mismatch)

Sequence: CGGTCATCAACTCGTTTCCTAGG

Oligo pair fwd: ATTGCGGTCATCAACTCGTTTCCT rev: AAACAGGAAACGAGTTGATGACCG

T218 out of 220

<Previous Next>

Sequence: CGGTCATCAACTCGTTTCCTAGG

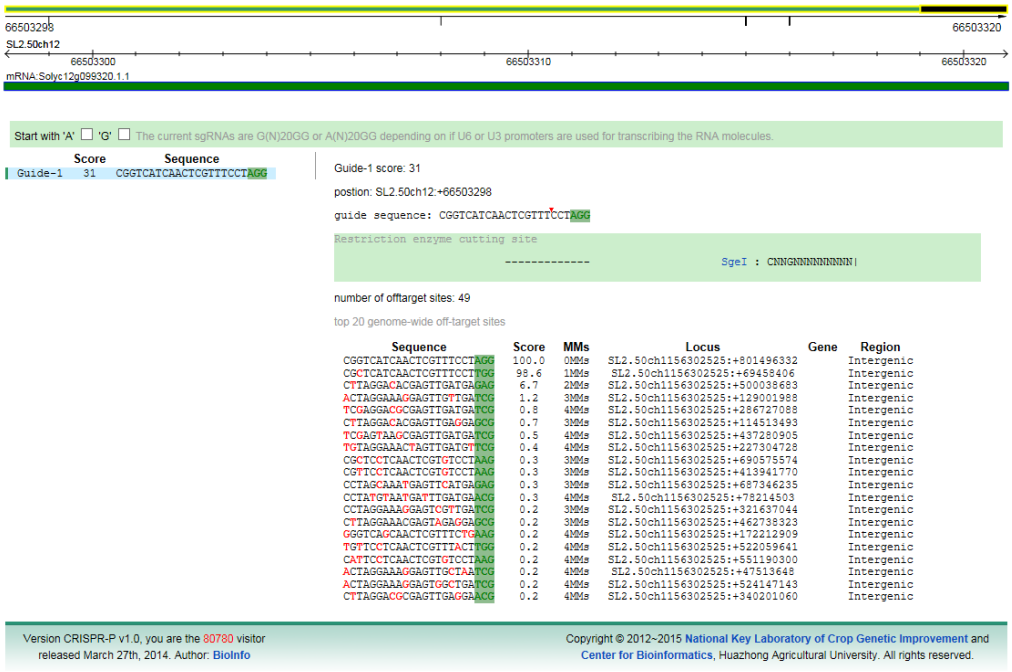
Oligo pair fwd: ATTGCGGTCATCAACTCGTTTCCT rev: AAACAGGAAACGAGTTGATGACCG

Coordinates	strand	MM	target_seq	PAM	distance		gene name	gene id
SL2.50ch12:66503298-66503320	+	0	CGGTCATC [AACTCGTTTCCT]	AGG	0	E	Serine/threonine-protein phosphatase 4 regulatory subunit 3 (AHRD V1 **-- PP4R3_DANRE); contains Interpro domain(s) IPR006887 Protein of unknown function DUF625	Solyc12g099320.1.1
SL2.50ch01:69458389-69458411	+	1	CGCTCATC [AACTCGTTTCCT]	TGG	0	E	Serine/threonine-protein phosphatase 4 regulatory subunit 3 (AHRD V1 **-- PP4R3_DANRE); contains Interpro domain(s) IPR006887 Protein of unknown function DUF625	Solyc01g060080.2.1

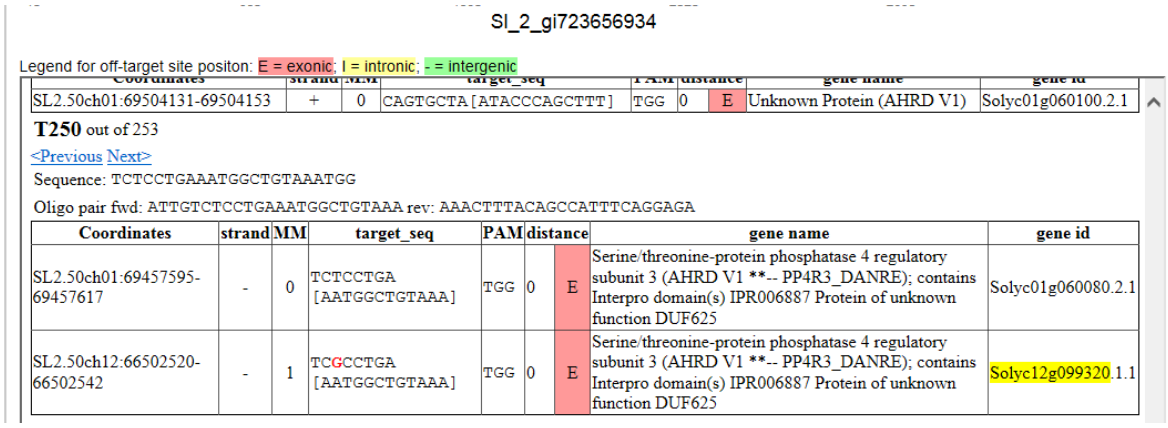
T219 out of 220

<Previous Next>

Sequence: GCTAGTTCAGTTGAATACTCCGG

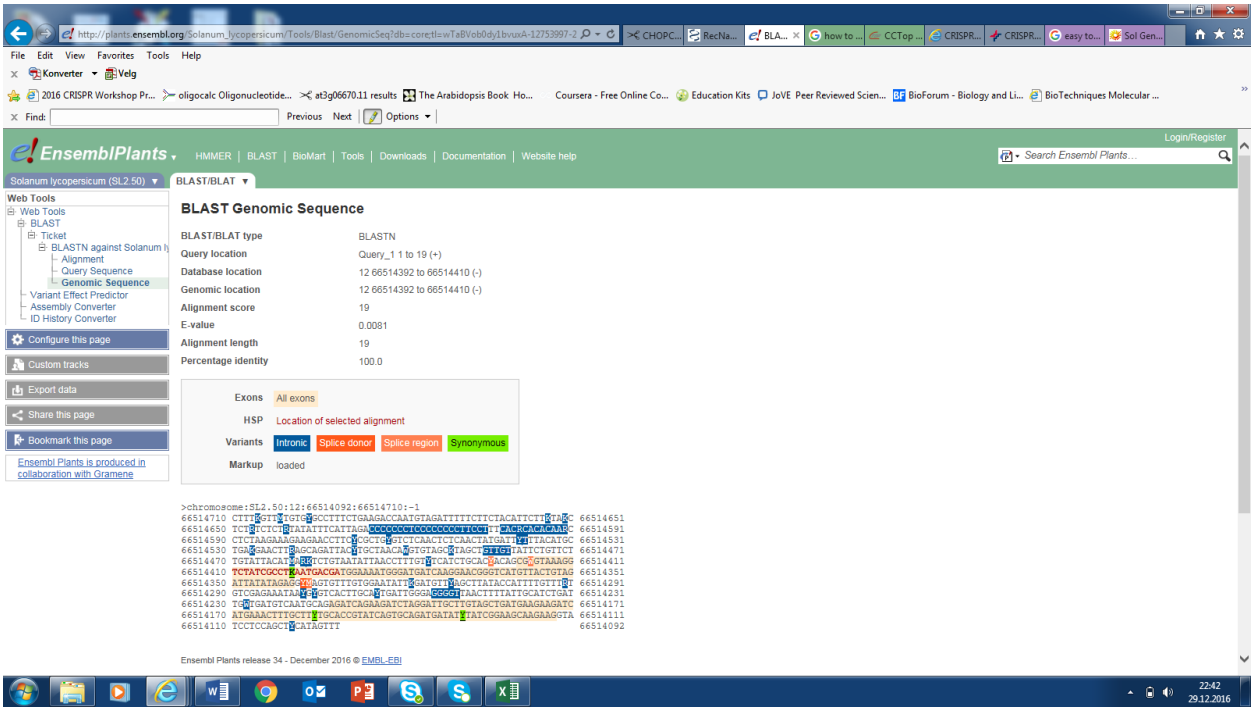


T250 out of 253 (has 12 off-targets with 4 mismatches)
Sequence: TCTCCTGAAATGGCTGTAAATGG
Oligo pair fwd: ATTGTCTCCTGAAATGGCTGTAAA rev: AAACTTTACAGCCATTTCAGGAGA

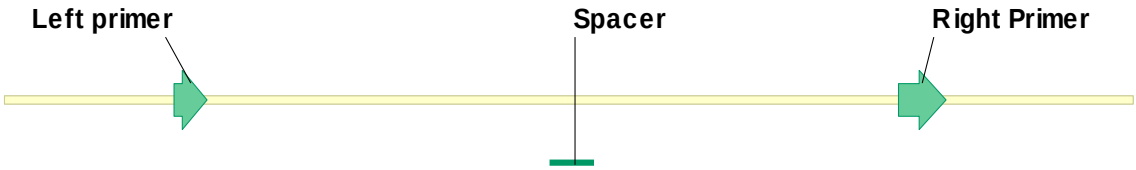


3.4.5.5 Searching for sgRNA places in tomato genome to be able to design flanking primers for PCR screening

For PSY2L-1 spacer 1



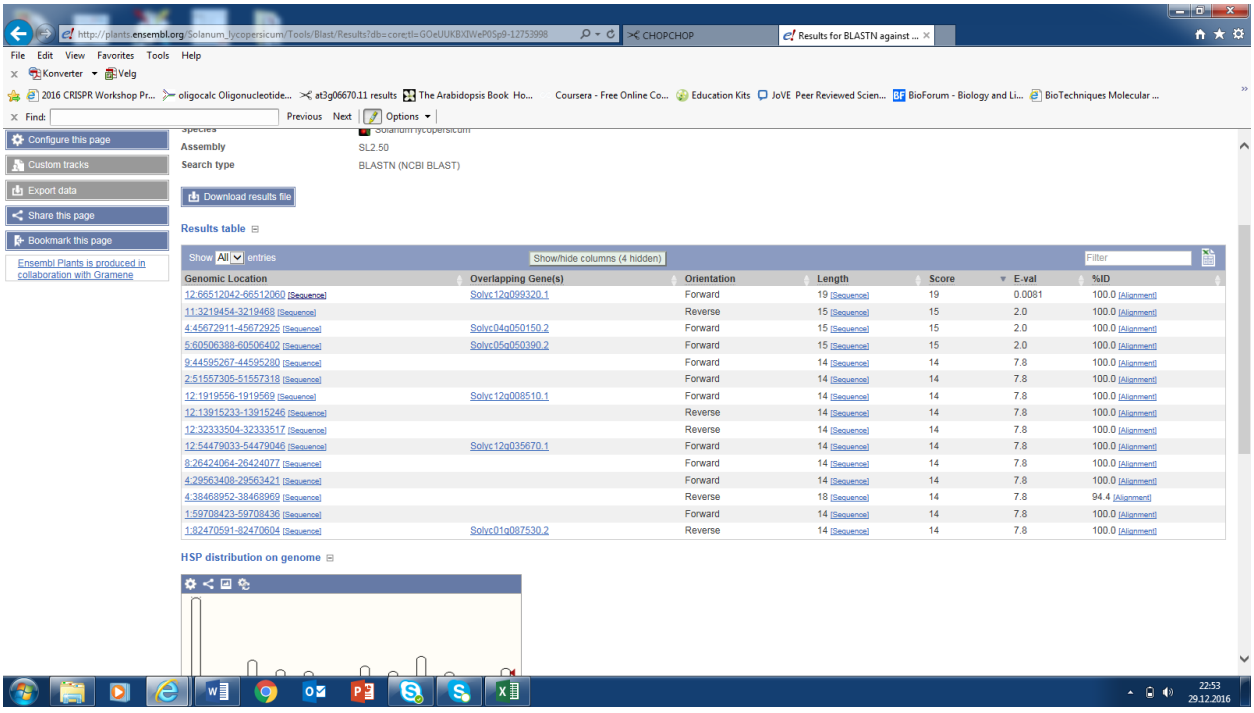

```
chromosome:SL2.50:12:66514092:66514710:-1
66514710 CTTTKGTTMTGTGYGCCTTTCTGAAGACCAATGTAGATTTTTCTTCTACATTCTTKTAKC
66514651
66514650 TCTRTCTCTRTATATTTCATTAGACCCCCCCTCCCCCCCCTTCCTTTCACRCACACAARC
66514591
66514590 CTCTAAGAAAGAAGAACCTTCYCGCTGYGTCTCAACTCTCAACTATGATTYTTTACATGC
66514531
66514530 TGAKGAACTTRAGCAGATTACYTGCTAACAWGTGTAGCKTAGCTGTTGTTATTCTGTTCT
66514471
66514470 TGTATTACATMARKTCTGTAATATTAACCTTTGTYTCATCTGCACYACAGCGWGTAAAGG
66514411
66514410 TCTATCGCCTKAATGACGATGGAAAATGGGATGATCAAGGAACGGGTCATGTTACTGTAG
66514351
66514350 ATTATATAGAGGYMAGTGTTTGTGGAATATTKGATGTTYAGCTTATACCATTTTGTTTRT
66514291
66514290 GTCGAGAAATAAYGYGTCACTTGCAYTGATTGGGAGGGGTTAACTTTTATTGCATCTGAT
66514231
66514230 TGWTGATGTCAATGCAGAGATCAGAAGATCTAGGATTGCTTGTAGCTGATGAAGAAGATC
66514171
66514170 ATGAAACTTTGCTTYTGCACCGTATCAGTGCAGATGATATYTATCGGAAGCAAGAAGGTA
66514111
66514110 TCCTCCAGCTYCATAGTTT
```

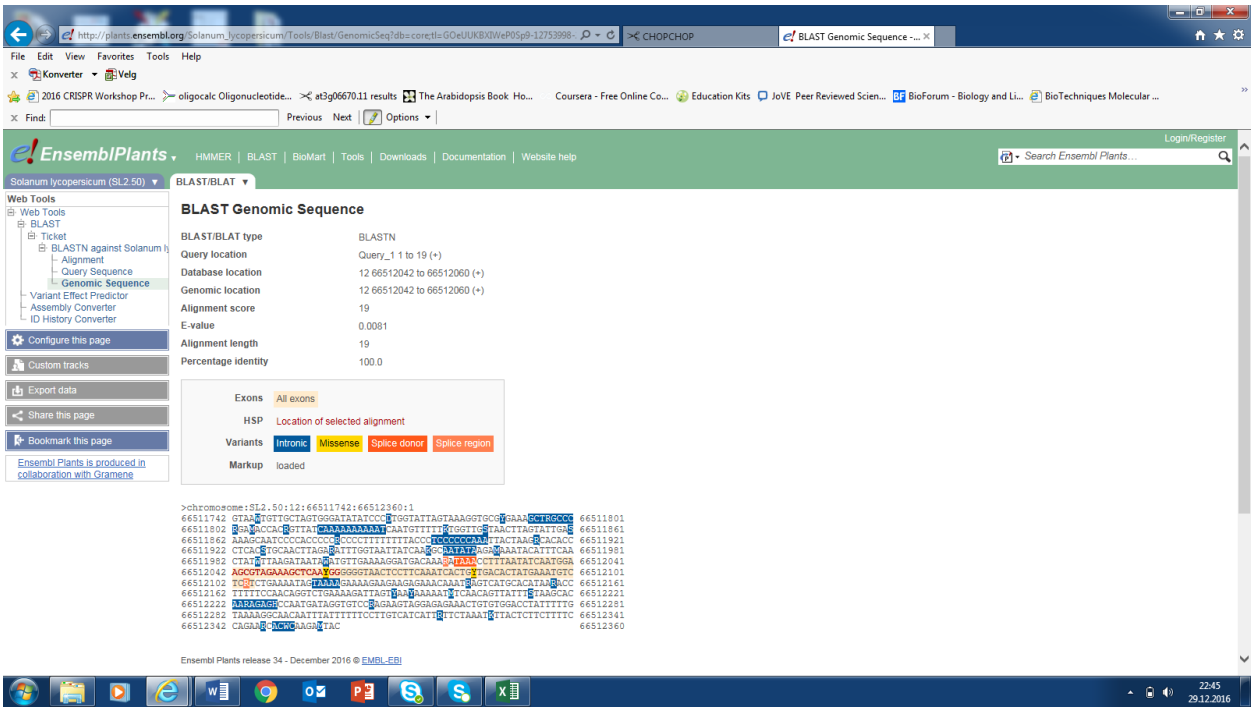


SLPSY2L-1 spacer1

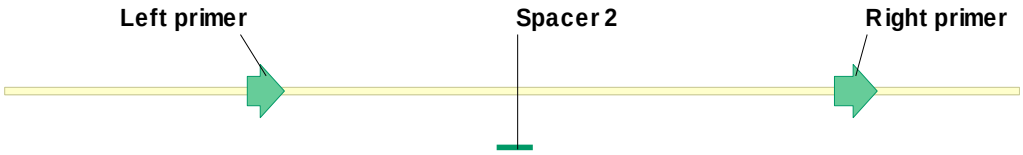
619 bp

FOR PSY2L-1 spacer 2





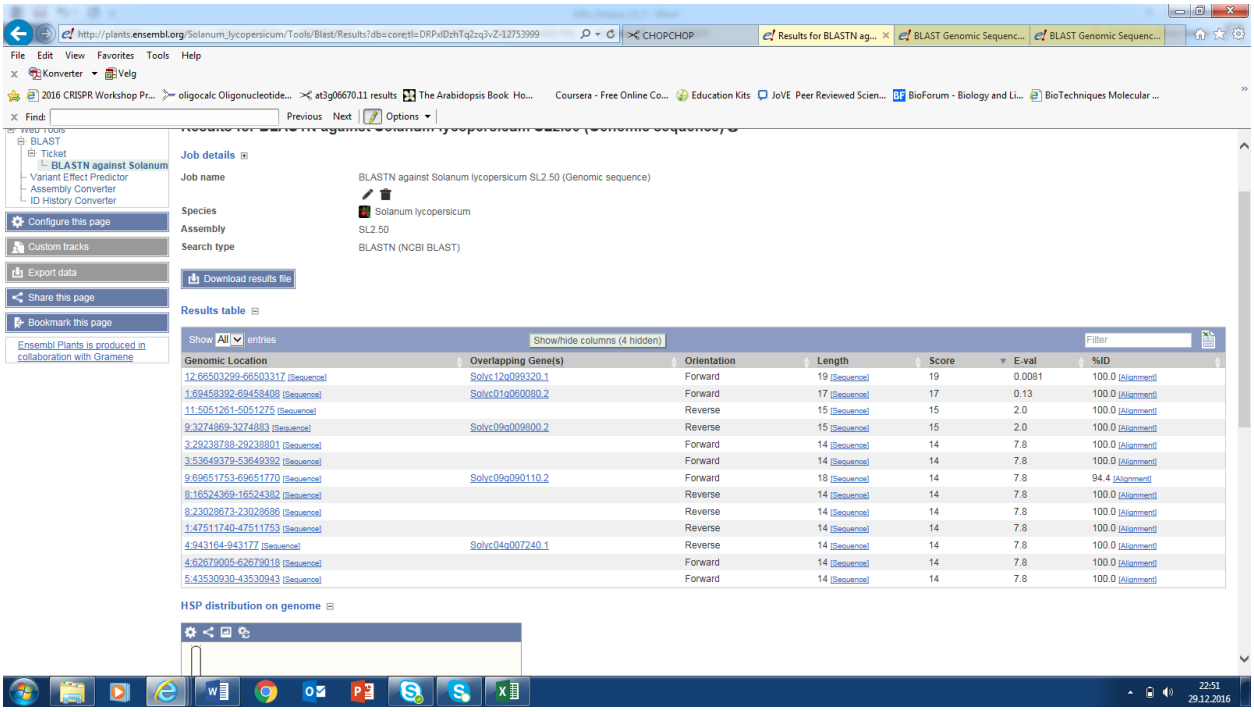

```
>chromosome:SL2.50:12:66511742:66512360:1
66511742 GTAAWTGTTGCTAGTGGGATATATCCCDTGGTATTAGTAAAGGTGCGYGAAAGCTRGCCC
66511801
66511802 RGAMACCACRGTTATCAAAAAAAAAATCAATGTTTTTKTGGTTGSTAACTTAGTATTGAS
66511861
66511862 AAAGCAATCCCCACCCCCRCCCCTTTTTTTTACCCTCCCCCCAAATTACTAAGRCACACC
66511921
66511922 CTCACSTGCAACTTAGARATTTGGTAATTATCAAKGCAATATAAGAMAAATACATTTCAA
66511981
66511982 CTATWTTAAGATAATAWATGTTGAAAAGGATGACAAARATAAACCTTTAATATCAATGGA
66512041
66512042 AGCGTAGAAAGCTCAAYGGGGGGGTAACTCCTTCAAATCACTGYTGACACTATGAAATGTC
66512101
66512102 TCRTCTGAAAATAGTAAAAGAAAAGAAGAAGAGAAACAAATRAGTCATGCACATAARACC
66512161
66512162 TTTTTCCAACAGGTCTGAAAAGATTAGTYAAYAAAAATMTCAACAGTTATTTSTAAGCAC
66512221
66512222 AARAGAGHCCAATGATAGGTGTCCRAGAAGTAGGAGAGAAACTGTGTGGACCTATTTTTG
66512281
66512282 TAAAAGGCAACAATTTATTTTTCCTTGTCATCATTRTTCTAAATKTTACTCTTCTTTTC
66512341
66512342 CAGAARCACWCAAGAMTAC
```

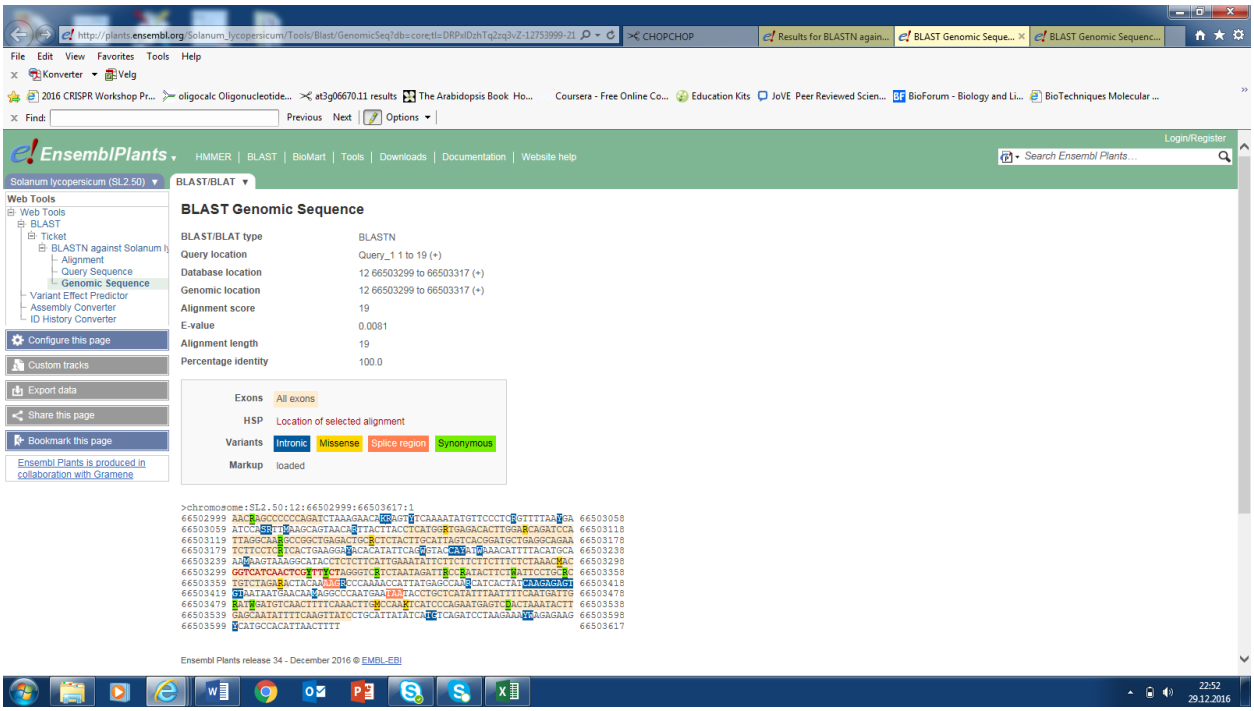


SlPSY2L-1 Spacer 2

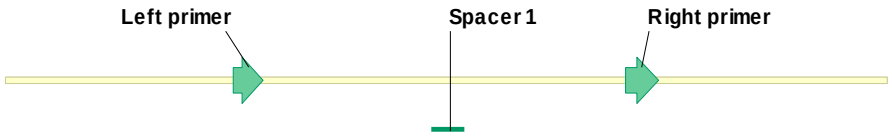
619 bp

For PSY2L1 and-2 spacer 1



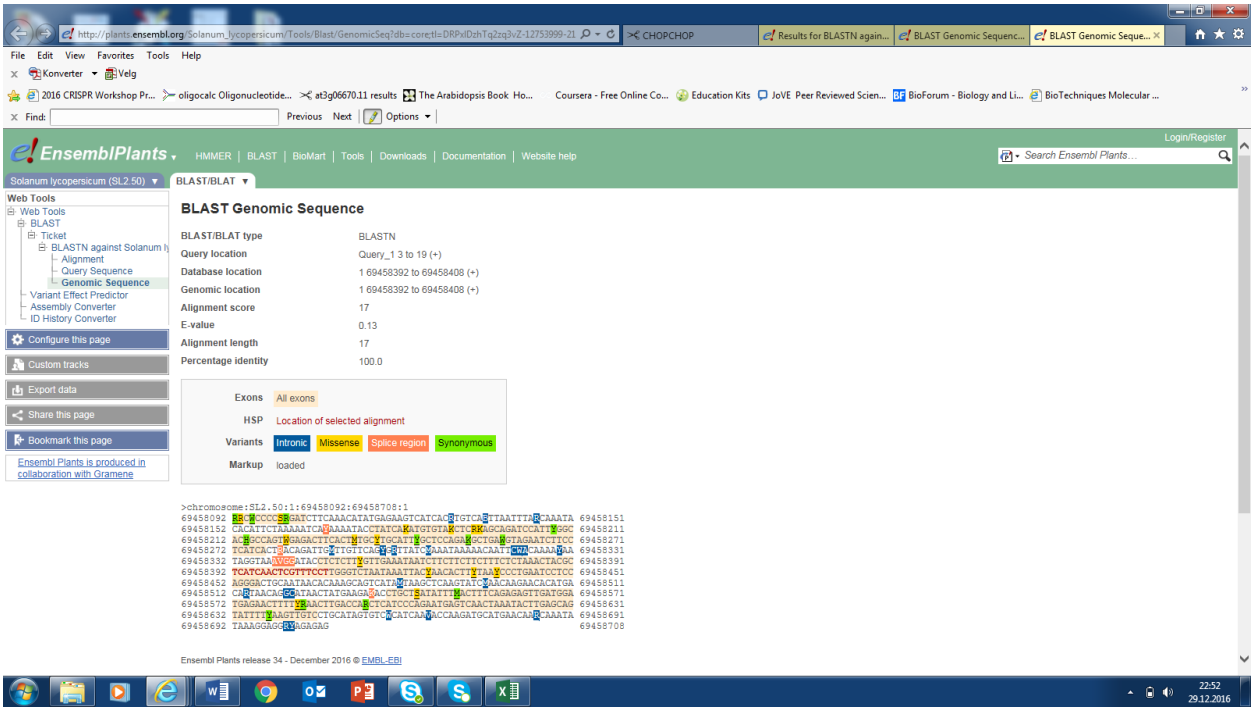



```
>chromosome:SL2.50:12:66502999:66503617:1
66502999 AACRAGCCCCCCAGATCTAAAGAACAKRAGTYTCAAAATATGTTCCCTCRGTTTTAAYGA
66503058
66503059 ATCCASRTTMAAGCAGTAACARTTACTTACCTCATGGRTGAGACACTTGGARCAGATCCA
66503118
66503119 TTAGGCAARGCCGGCTGAGACTGCRCTCTACTTGCATTAGTCACGGATGCTGAGGCAGAA
66503178
66503179 TCTTCCTCRTCACTGAAGGAYACACATATTCAGWGTACCAYATWAAACATTTTACATGCA
66503238
66503239 AAMAAGTAAAGGCATACCTCTCTTCATTGAAATATTCTTCTTCTTCTTTCTCTAAACMAC
66503298
66503299 GGTCATCAACTCGYTTYCTAGGGTCRTCTAATAGATTRCCRATACTTCTWATTCCTGCRC
66503358
66503359 TGTCTAGARACTACAAAAGRCCCAAAACCATTATGAGCCAARCATCACTATCAAGAGAGT
66503418
66503419 GTAATAATGAACAAMAGGCCCAATGAATAATACCTGCTCATATTTAATTTTCAATGATTG
66503478
66503479 RATWGATGTCAACTTTTCAAACTTGMCCAAKTCATCCCAGAATGAGTCDACTAAATACTT
66503538
66503539 GAGCAATATTTTCAAGTTATCCTGCATTATATCATGTCAGATCCTAAGAAAYWAGAGAAG
66503598
66503599 YCATGCCACATTAACTTTT
```



SlPSY2L-1-2-Spacer 1

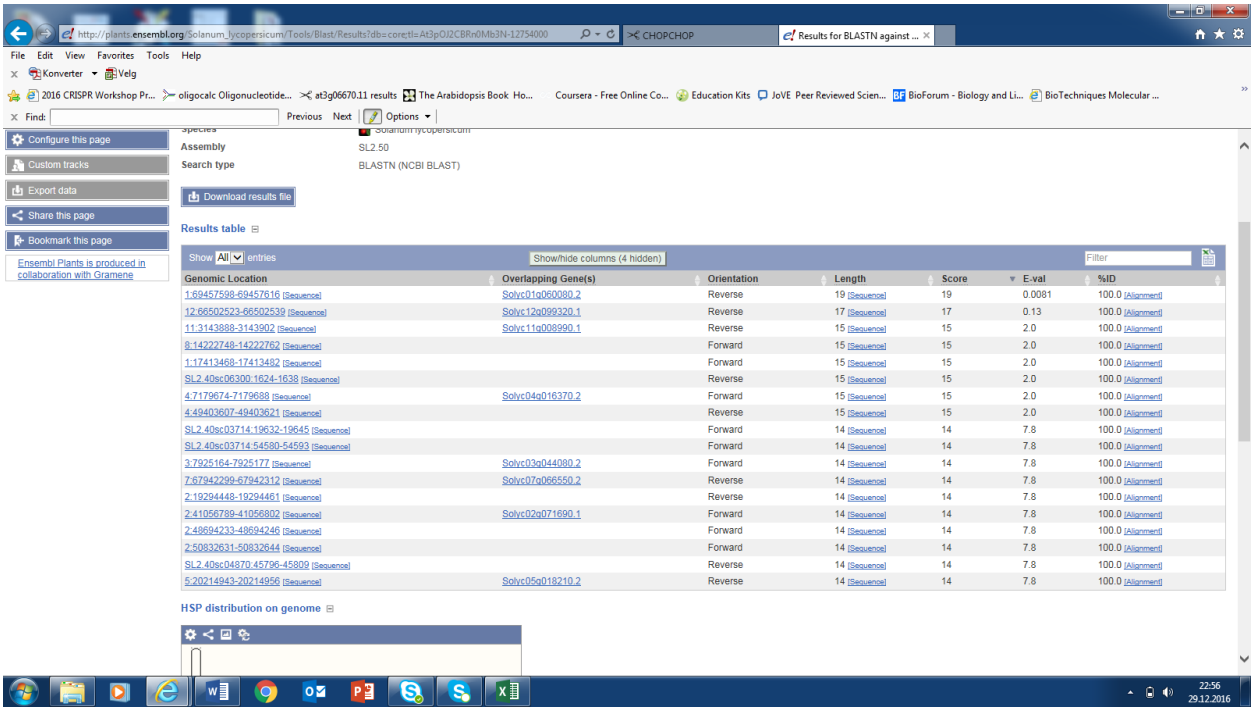
619 bp

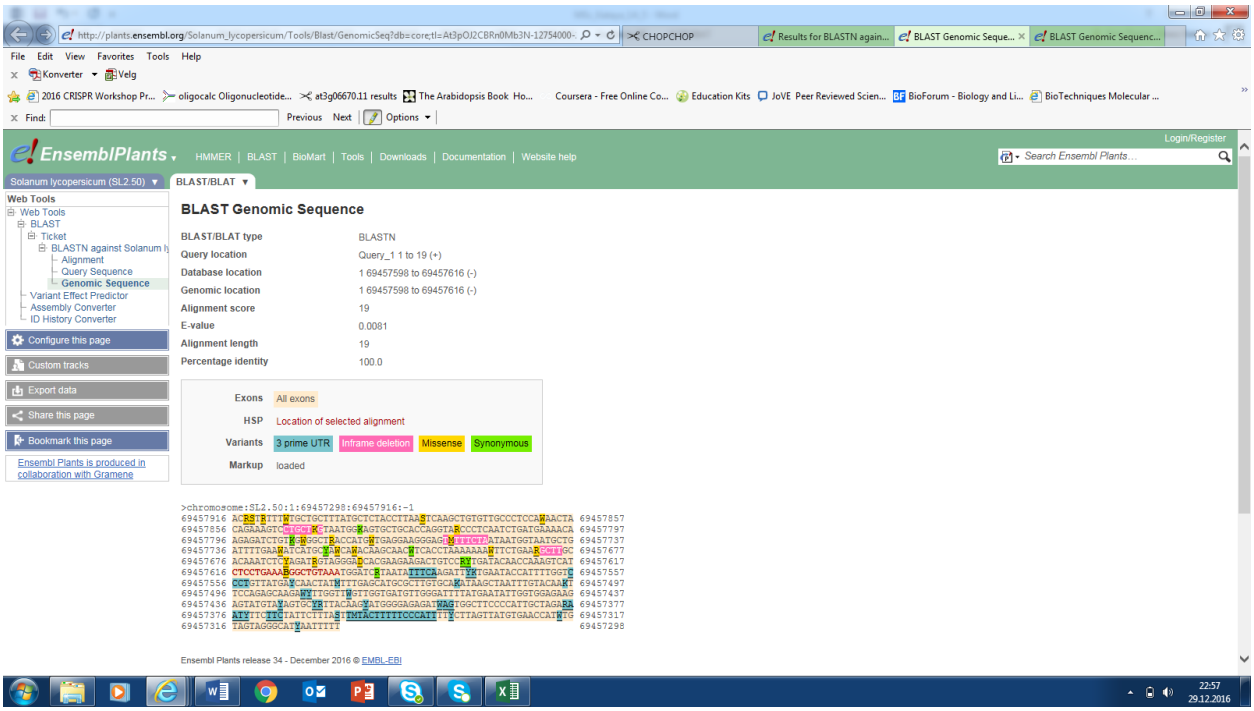



```
chromosome:SL2.50:1:69458092:69458708:1
69458092 RRCWCCCCSRGATCTTCAAACATATGAGAAGTCATCACRTGTCABTTAATTTARCAAATA
69458151
69458152 CACATTCTAAAAATCAYAAAATACCTATCAKATGTGTAKCTCRKAGCAGATCCATTYGGC
69458211
69458212 ACHGCCAGTWGAGACTTCACTMTGCYTGCATTYGCTCCAGAKGCTGAWGTAGAATCTTCC
69458271
69458272 TCATCACTRACAGATTGMTTGTTCAGYGRTTATCMAAATAAAAACAATTCWACAAAAYAA
69458331
69458332 TAGGTAAAVGCATACCTCTCTTYGTTGAAATAATCTTCTTCTTCTTTCTCTAAACTACGC
69458391
69458392 TCATCAACTCGTTTCCTTGGGTCTAATAAATTACYAACACTTYTAAYCCCTGAATCCTCC
69458451
69458452 AGGGACTGCAATAACACAAAGCAGTCATAMTAAGCTCAAGTATCMAACAAGAACACATGA
69458511
69458512 CARTAACAGGCATAACTATGAAGAKACCTGCTSATATTTMACTTTCAGAGAGTTGATGGA
69458571
```

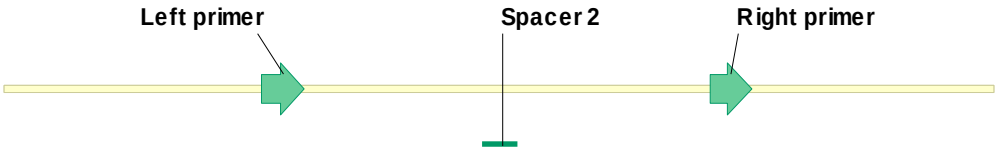
69458572 TGAGAACTTTTYRAACTTGACCARCTCATCCCAGAATGAGTCAACTAAATACTTGAGCAG
69458631
69458632 TATTTTYAAGTTGTCCTGCATAGTGTCWCATCAAVACCAAGATGCATGAACAARCAAATA
69458691
69458692 TAAAGGAGGRYAGAGAG

For PSY2L1 and-2 spacer 2



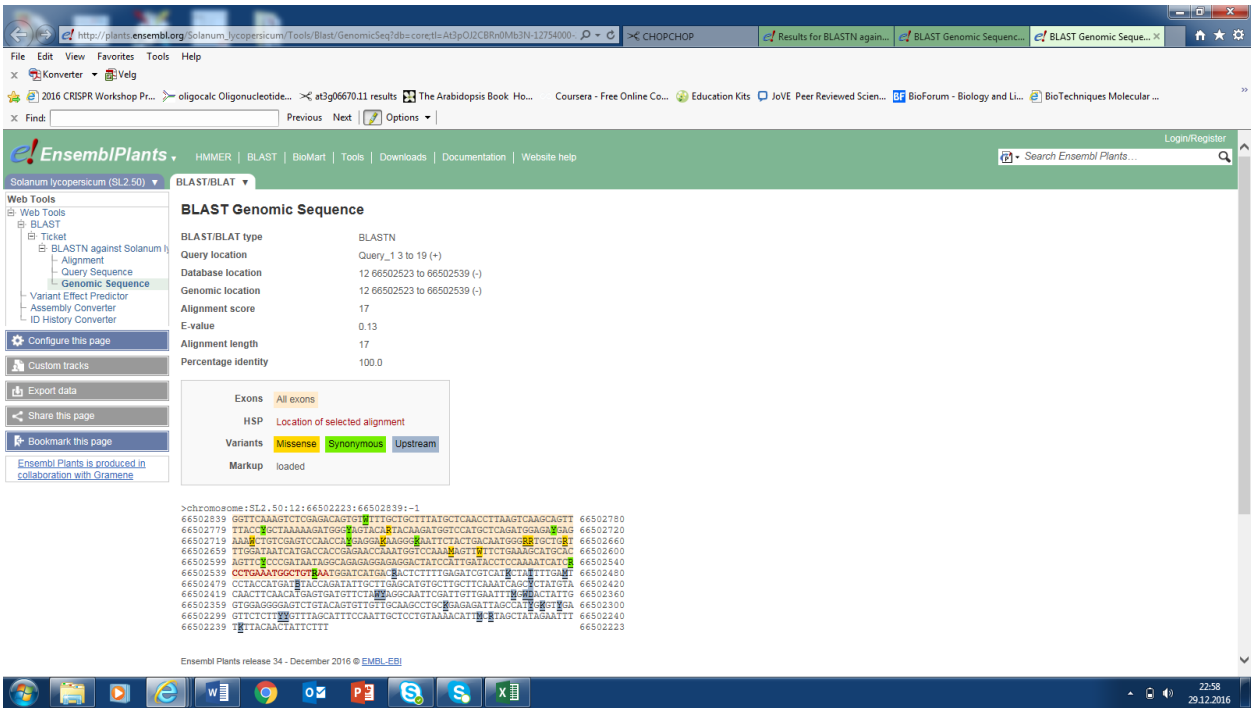



```
>chromosome:SL2.50:1:69457298:69457916:-1
69457916 ACRSTRTTTWTGCTGCTTTATGCTCTACCTTAASTCAAGCTGTGTTGCCCTCCAWAACTA
69457857
69457856 CAGAAAGTCCTGCTKGTAATGGKAGTGCTGCACCAGGTARCCCTCAATCTGATGAAAACA
69457797
69457796 AGAGATCTGTKGWGGCTRACCATGWTGAGGAAGGGAGTMTTTCTAATAATGGTAATGCTG
69457737
69457736 ATTTTGAAWATCATGCYAWCAWACAAGCAACWTCACCTAAAAAAAWTTCTGAARGCTTGC
69457677
69457676 ACAAATCTCYAGATRGTAGGGADCACGAAGAAGACTGTCCRYTGATACAACCAAAGTCAT
69457617
69457616 CTCCTGAAABGGCTGTAAATGGATCRTAATATTTCAAGATTYKTGAATACCATTTTGGTC
69457557
69457556 CCTGTTATGAYCAACTATMTTTGAGCATGCGCTTGTGCAKATAAGCTAATTTGTACAAKT
69457497
69457496 TCCAGAGCAAGAWYTTGGTTWGTTGGTGATGTTGGGATTTTATGAATATTGGTGGAGAAG
69457437
69457436 AGTATGTAYAGTGCYRTTACAAGYATGGGGAGAGATWAGTGGCTTCCCCATTGCTAGARA
69457377
69457376 ATYTTCTTCTATTCTTTASTTMTACTTTTTCCCATTTTYCTTAGTTATGTGAACCATWTG
69457317
69457316 TAGTAGGGCATYAATTTTT
```



Sl-PSY2L-2-1-Spacer 2

619 bp

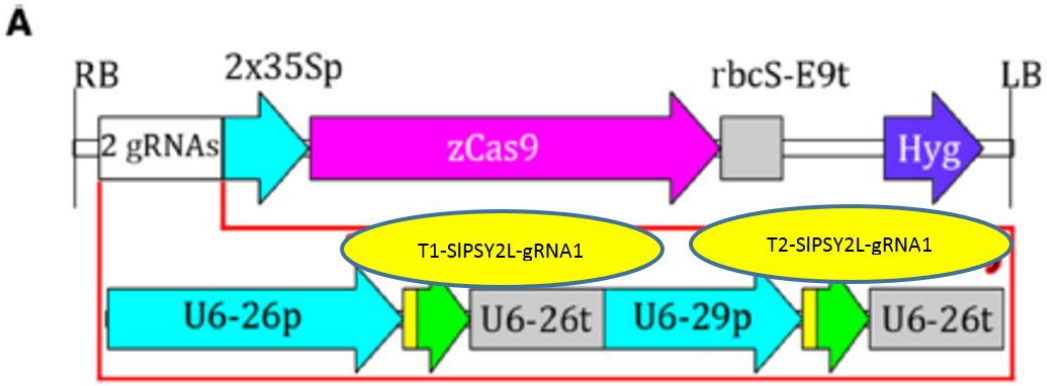



```
>chromosome:SL2.50:12:66502223:66502839:-1
66502839 GGTTCAAAGTCTCGAGACAGTGTWTTTGCTGCTTTATGCTCAACCTTAAGTCAAGCAGTT
66502780
66502779 TTACCYGCTAAAAAGATGGGYAGTACARTACAAGATGGTCCATGCTCAGATGGAGAYGAG
66502720
66502719 AAAWCTGTCGAGTCCAACCAYGAGGAKAAGGGKAATTCTACTGACAATGGGRRTGCTGRT
66502660
66502659 TTGGATAATCATGACCACCGAGAACCAAATGGTCCAAAMAGTTWTTCTGAAAGCATGCAC
66502600
66502599 AGTTCYCCCGATAATAGGCAGAGAGGAGAGGACTATCCATTGATACCTCCAAAATCATCR
66502540
66502539 CCTGAAATGGCTGTRAATGGATCATGACRACTCTTTTGAGATCGTCATKCTATTTTGAMT
66502480
66502479 CCTACCATGATBTACCAGATATTGCTTGAGCATGTGCTTGCTTCAAATCAGCYCTATGTA
66502420
```

```
66502419 CAACTTCAACATGAGTGATGTTCTAWYAGGCAATTCGATTGTTGAATTTMGWDACTATTG
66502360
66502359 GTGGAGGGGAGTCTGTACAGTGTTGTTGCAAGCCTGCKGAGAGATTAGCCATYGKGTYGA
66502300
66502299 GTTCTCTTYYGTTTAGCATTTCCAATTGCTCCTGTAAAACATTMCRTAGCTATAGAATTT
66502240
66502239 TKTTACAACTATTCTTT
```

3.4.6 Amplifying of one or two gRNA expression cassette including one or two Sl-PSY2L spacers from the vector pCBC-DT1DT2

The method of cloning of gRNA into binary vector depends on two different strategies that were described (Xing et al. 2014) as in the snapshot bellow:



carrying one or more gRNAs for targeted mutations of multiple plant genes. This toolkit, which facilitates transient or stable expression of CRISPR/Cas9 in a variety of plant systems, can be applied to a variety of plants and is especially useful for high-efficiency generation of mutants bearing multiple gene mutations.

Methods

Vector construction

Detailed descriptions of the vector construction are provided in Additional file 2: Methods S1. All primers used in this report are listed in Additional file 1: Table S1.

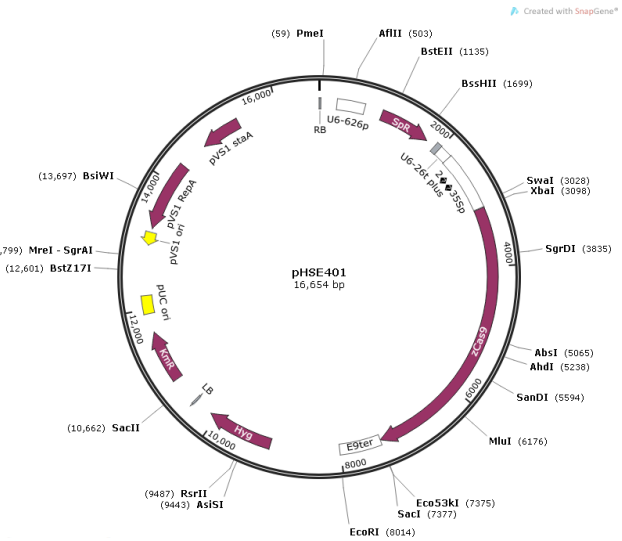
Golden gate method to construct a vector expressing one or two gRNAs

For assembly of one gRNA, equal volumes of 100 μmol/L oligos 1 and 2 were mixed, incubated at 65°C for 5 minutes, and cooled slowly to room temperature, resulting in a double-stranded insert with 4-nt 5′ overhangs at both ends. For assembly of two gRNAs, the two target sites were incorporated into PCR forward and reverse primers, respectively. The PCR fragment was amplified from pCBC-DT1T2 for dicot targets or pCBC-MT1T2 for monocot targets with two long primers or four shorter primers, among which two forward or two reverse primers were partially overlapping. The insert or the purified PCR fragment (T1T2-PCR), together with any of the binary vectors described in this report, were used to set up restriction-ligation reactions, as described elsewhere [44], using *Bsa*I and T4 Liga s (New England Biolabs). The reaction was incubated in a thermocycler for 5 hours at 37°C, 5 min at 50°C and 10 min at 80°C. Detailed information including gRNA module sequences, PCR primers, colony PCR primers, and sequencing primers can be found in Additional file 3: Methods S2.

We are planning to incorborate two gRNAs in one step, also to two clone each of the selected gRNAs alone. we will use two different ways of cloning:

1- Cloning of each of spacers solely by direct cloning into the binary vector pHSE401 (described in red in the above text). This will need the addition of BsaI 4 nt overhangs in the primers. This step means two direct cloning of pHSE401-gRNA1 and pHSE401-gRNA1 vectors
2- Incorporating two gRNAs in one expression cassette simultaneously by PCR amplification by 4 different primer (includes the two different spacers) using the vector pCBC-DT1DT2 as a template…(Highlighted in blue). This is followed by subcloning into the binary vector pHSE401 through BsaI digestion and ligation.

3.4.6.1 Golden Gate cloning method for the assembly of one or two gRNAs



Simplified protocol

1. Manually search for 23-bp target sites (5'-N_{20}NGG-3') within exons of sequences of genes of interest, and then evaluate target specificities by potential off-target finder (http://www.rgenome.net/cas-offinder/). Find target sites on the website of genome-wide prediction of plant CRISPR (http://www.genome.arizona.edu/crispr/CRISPRsearch.html).
2. Design primers:
   a) Find names of inserts/oligos (for one gRNA) or PCR fragments according to plant species (monocots or dicots) and gRNA number
   b) Find the sequences of the oligos/primers according to the name
   c) Replace 19-nt N in the forward primers with your 19-nt target PAM (NGG), and 19-nt N in the reverse primers with reverse complement your 19-nt target sequences in front of PAM (NGG).
3. Carry out PCR reactions according to information provided under the sequences of the PCR fragments. As an example, the reaction mixture and reaction conditions are as follows for construction of pHSE401-2gR-CHLI:

Component	Volume	Cycling conditions
10× KOD plus Buffer	5 µl	
MgSO4 (25mM)	3 µl	
dNTPs (2mM, Toyobo)	4 µl	
KOD plus (Toyobo)	1 µl	
pCBC-DT1T2 (diluted to 200 times)	1 µl	1. One cycle: 94 °C, 2 min.
DT1-BsF (20 µM)	1 µl	2. 30 cycles: 94 °C, 15 sec; 60 °C, 30 sec; 68 °C, 1 min.
DT1-F0 (1 µM)	1 µl	3. One cycle: 68 °C, 5 min
DT2-R0 (1 µM)	1 µl	
DT2-BsR (20 µM)	1 µl	
ddH_2O	32 µl	
Total volume	50 µl	

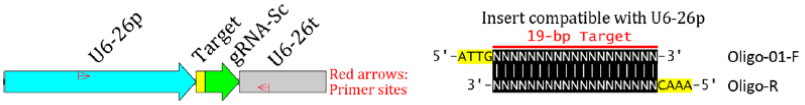
4. Set up Golden Gate reactions as follows:

Component	Volume	Reaction conditions
Purified PCR framents (~100 ng/µl)	2 µl	5 hours at 37°C 5 min at 50°C 10 min at 80°C NOTE: It is essential to use a High Concentration (HC) Ligase (2 million units/ml, NEB)
pHSE401 or others (~100 ng/µl)	2 µl	
10× T4 DNA Ligase Buffer (NEB)	1.5 µl	
10× BSA	1.5 µl	
*Bsa*I (NEB)	1 µl	
T4 DNA Ligase (HC, NEB)	1 µl	
ddH_2O	6 µl	
Total volume	15 µl	

5. Transform *E.coli* competent cells with 5 µl of reaction mixture, and select positive clones on kanamycin LB agar plates.
6. Identify correct clones by colony PCR and verify them by sequencing.

3.4.6.2 Sequence of one gRNA expression cassette for dicots

23-bp insert + pHSN401 et al.



(U6-26p)-(Target-1)-(gRNA-Sc)-(U6-26t)

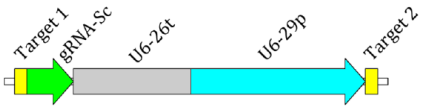
CGACTTGCCTTCCGCACAATACATCATTTCTTCTTAGCTTTTTTTCTTCTTCTTCGTTCATACAGTTTTTTTTTGT
TTATCAGCTTACATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTTAACTTTCCATTCGGAGTTTTTGTATCTT
GTTTCATAGTTTGTCCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTT
CATTCTTAAGATATGAAGATAATCTTCAAAAGGCCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATA
TGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTT
AGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTGNNNNNNNNNNNNNNNN
NNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGA
GTCGGTGCTTTTTTTTGCAAAATTTTCCAGATCGATTTCTTCTTCCTCTGTTCTTCGGCGTTCAATTTCTGGGG
TTTTCTCTTCGTTTTCTGTAACTGAAACCTAAAATTTGACCTAAAAAAAATCTCAAATAATATGATTCAGTGGTT
TTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCCGATGAGATAAACCAATA

Notes:

1. Underlined letters come from binary vectors, while the others come from PCR fragments.
2. Red letters indicate primer sites.
3. Primer sequences are as follows

Colony PCR primers (5'→3'):
U6-26p-F: TGTCCCAGGATTAGAATGATTAGGC U6-26t-R: CCCCAGAAATTGAACGCCGAAGAAC
(U6-26p-F + U6-26t-R = 423 bp)
Sequencing primers (5'→3'): U6-26p-F: TGTCCCAGGATTAGAATGATTAGGC

3.4.6.3 Sequence of DT1T2-PCR with Targets 1 and 2 for dicots



ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
GCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGCAAAATTTTCCAGATCGATTT
CTTCTTCCTCTGTTCTTCGGCGTTCAATTTCTGGGGTTTTCTCTTCGTTTTCTGTAACTGAAACCTAAAATTTG
ACCTAAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCCGAT
GAGATAAACCAATATTAATCCAAACTACTGCAGCCTGACAGACAAATGAGGATGCAAACAATTTTAAAGTTT
ATCTAACGCTAGCTGTTTTGTTTCTTCTCTCTGGTGCACCAACGACGGCGTTTTCTCAATCATAAAGAGGCT
TGTTTTACTTAAGGCCAATAATGTTGATGGATCGAAAGAAGAGGGCTTTTAATAAACGAGCCCGTTTAAG
CTGTAAACGATGTCAAAAACATCCCACATCGTTCAGTTGAAAATAGAAGCTCTGTTTATATATTGGTAGAG
TCGACTAAGAGATTGNNNNNNNNNNNNNNNNNNGTTTAGAGACCAATAAT

Primers:
DT1-BsF: ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNGTT
DT1-F0: TGNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC
DT2-R0: AACNNNNNNNNNNNNNNNNNNCAATCTCTTAGTCGACTCTAC
DT2-BsR: ATTATTGGTCTCGAAACNNNNNNNNNNNNNNNNNNCAA
Template: pCBC-DT1T2
Length: 626-bp
Notes:

4. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
5. For the assembly of two gRNA expression cassettes, use DT1-BsF/DT1-F0/DT2-R0/DT2-BsR

four-primer mixture with DT1-F0/DT2-R0 diluted to 20 times of DT1-BsF or DT2-BsR, resulting in DT1T2-PCR.

3.4.6.4 template from the article for designing primers of dual targeting by golden gate

The template for primer design is in the previous section 3.4.6.3

This is an example, colored by me to confirm the understanding of cloning.. The new gRNAs (19 bp) will be added in their places. gRNA1 will be only forward added, while the gRNA2 will be reverse added.

DT1A-BsF/TC AATAATGGTCTCTATTGAATATCTCTCTATCTCCTCGTT
DT1A-F0/TC TGAATATCTCTCTATCTCCTCGTTTTAGAGCTAGAAATAGC
DT2-R0/ETC2 AACATTCGATGCTACTCACTTCCAATCTCTTAGTCGACTCTAC
DT2-BsR/ETC2 ATTATTGGTCTCTAAACATTCGATGCTACTCACTT

3.4.6.5 Primers for generating 2 gRNAs against Sl-PSY2L 1

From section 3.4.5.1 gRNA1 (19-PAM): TCTATCGCCTGAATGACGA *to be used in Forward primers*
gRNA2: AGCGTAGAAAGCTCAATGG
gRNA2 reverse: CCATTGAGCTTTCTACGCT *to be used in reverse primers*

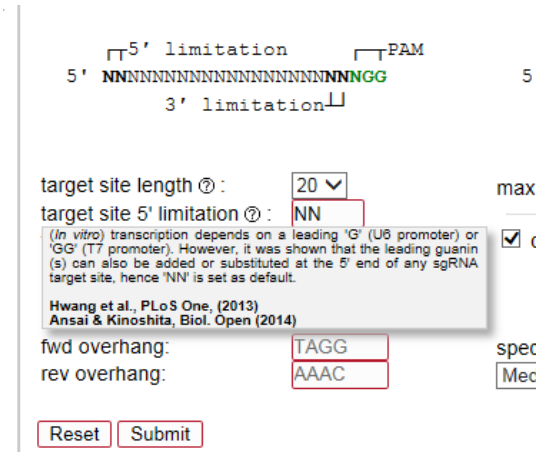
T1-BsF/PSY-1 AATAATGGTCTCTATTGTCTATCGCCTGAATGACGAGTT
T1-F0/PSY-1 TGTCTATCGCCTGAATGACGAGTTTTAGAGCTAGAAATAGC
T2-R0/PSY-1 AACCCATTGAGCTTTCTACGCTCAATCTCTTAGTCGACTCTAC
T2-BsR/PSY-1 ATTATTGGTCTCTAAACCCATTGAGCTTTCTACGCTC

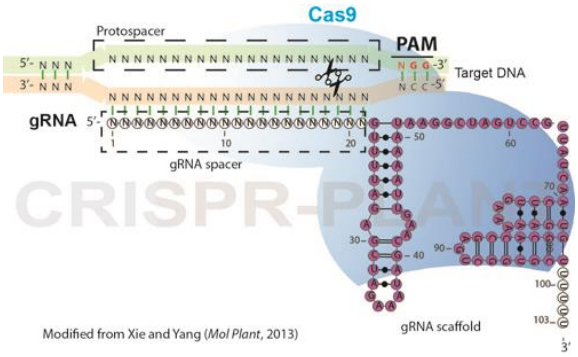
3.4.6.6 Primers for generating 2 gRNAs dual targeting both Sl-PSY2L 1 and 2

From section 3.4.5.4 gRNA1: GGTCATCAACTCGTTTCCT *to be used in Forward primers*
gRNA2: CTCCTGAAATGGCTGTAAA
gRNA2 reverse: TTTACAGCCATTTCAGGAG *to be used in reverse primers*

T1-BsF/PSY-1/2 AATAATGGTCTCTATTGGGTCATCAACTCGTTTCCTGTT
T1-F0/PSY-1/2 TGGGTCATCAACTCGTTTCCTGTTTTAGAGCTAGAAATAGC
T2-R0/PSY-2/1 AACTTTACAGCCATTTCAGGAGCAATCTCTTAGTCGACTCTAC
T2-BsR/PSY-2/1 ATTATTGGTCTCTAAACTTTACAGCCATTTCAGGAGC

After finishing design… copy gRNA spacer and check for off-targets on RGEN TOOLs…..





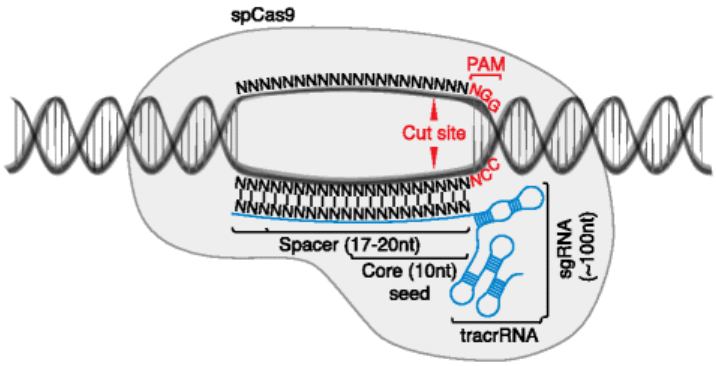


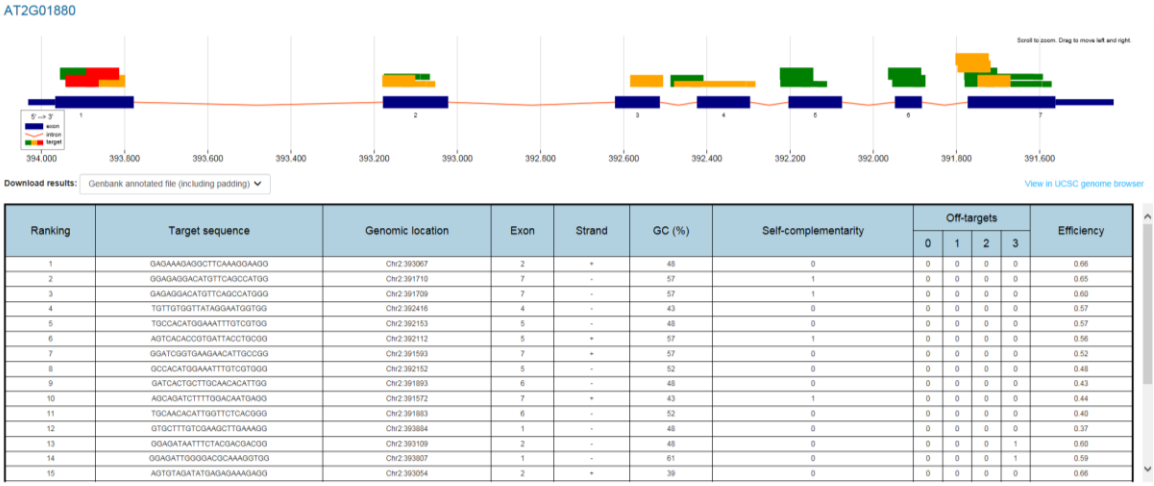
Fig. 1
Components of the CRISPR-Cas9 system. *Streptococcus pyogenes* Cas9 (*Sp*Cas9) forms a complex with a chimeric single guide RNA (*sgRNA*) comprising a spacer that hybridizes with the genomic target site, and a scaffold RNA termed *tracrRNA* required for complex formation. The protospacer adjacent motif (*PAM*) is required for sequence

https://genomebiology.biomedcentral.com/articles/10.1186/s13059-015-0823-x

3.5 Editing Purple acid phosphatase 7 and its near homolog using CRISPR in Arabidopsis

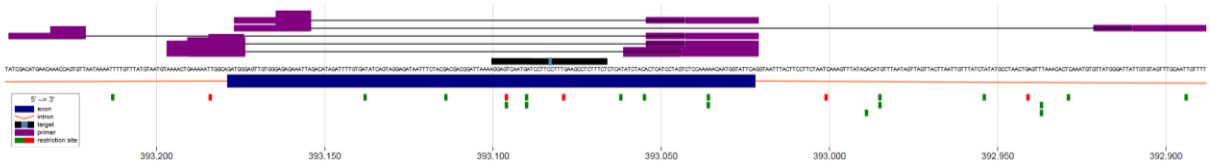
3.5.1 PAP7 and PAP?? Homology and peroxisomal identification

3.5.2 Predicting PAPs spacers



Ranking	Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
							0	1	2	3	
1	GAGAAAGAGGCTTCAAAGGAAGG	Chr2:393067	2	+	48	0	0	0	0	0	0.66
2	GGAGAGGACATGTTCAGCCATGG	Chr2:391710	7	-	57	1	0	0	0	0	0.65
3	GAGAGGACATGTTCAGCCATGGG	Chr2:391709	7	-	57	1	0	0	0	0	0.60
4	TGTTGTGGTTATAGGAATGGTGG	Chr2:392416	4	-	43	0	0	0	0	0	0.57
5	TGCCACATGGAAATTTGTCGTGG	Chr2:392153	5	-	48	0	0	0	0	0	0.57
6	AGTCACACCGTGATTACCTGCGG	Chr2:392112	5	+	57	1	0	0	0	0	0.56
7	GGATCGGTGAAGAACATTGCCGG	Chr2:391593	7	+	57	0	0	0	0	0	0.52
8	GCCACATGGAAATTTGTCGTGGG	Chr2:392152	5	-	52	0	0	0	0	0	0.48
9	GATCACTGCTTGCAACACATTGG	Chr2:391893	6	-	48	0	0	0	0	0	0.43
10	AGCAGATCTTTTGGACAATGAGG	Chr2:391572	7	+	43	1	0	0	0	0	0.44
11	TGCAACACATTGGTTCTCACGGG	Chr2:391883	6	-	52	0	0	0	0	0	0.40
12	GTGCTTTGTCGAAGCTTGAAAGG	Chr2:393884	1	-	48	0	0	0	0	0	0.37
13	GGAGATAATTTCTACGACGACGG	Chr2:393109	2	-	48	0	0	0	0	1	0.60
14	GGAGATTGGGGACGCAAAGGTGG	Chr2:393807	1	-	61	0	0	0	0	1	0.59
15	AGTGTAGATATGAGAGAAAGAGG	Chr2:393054	2	+	39	0	0	0	0	0	0.66

Spacer 1



Gene specific part of sgRNA
GAGAAAGAGGCTTCAAAGGAAGG exon 2
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr2:393176-393198	AAACTGAAAAATTGGCAGATGG	60.3	0	Chr2:393022-393045	CCTGAATACCATTGTTTTTGGAG	59.8	0	0	176
2	Chr2:393176-393198	AAACTGAAAAATTGGCAGATGG	60.3	0	Chr2:393022-393044	CCTGAATACCATTGTTTTTGGA	58.9	0	0	176
3	Chr2:393223-393245	TCGACATGAACAAACCAGTGTT	60.5	0	Chr2:393022-393044	CCTGAATACCATTGTTTTTGGA	58.9	0	0	223
4	Chr2:393156-393178	GGGAGTTGTGGGAGAGAAATTA	59.5	0	Chr2:392888-392911	CCAAAACAATTGCAAACTACACA	60.0	0	0	290
5	Chr2:393156-393178	GGGAGTTGTGGGAGAGAAATTA	59.5	0	Chr2:393022-393044	CCTGAATACCATTGTTTTTGGA	58.9	0	0	156

Chr2:393066-393086	4	3	GAGAAAGAGGCTTCAAAGGA	AGGATCATTG	+	exon

gRNA (Spacer was shown in upper-case):
5'-GAGAAAGAGGCTTCAAAGGAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GAGAAAGAGGCTTCAAAGGA-3'
5'-TCCTTTGAAGCCTCTTTCTC-3'

GC content of Spacer sequence: 0.45

Potential Pol III terminator (TTTTT): null

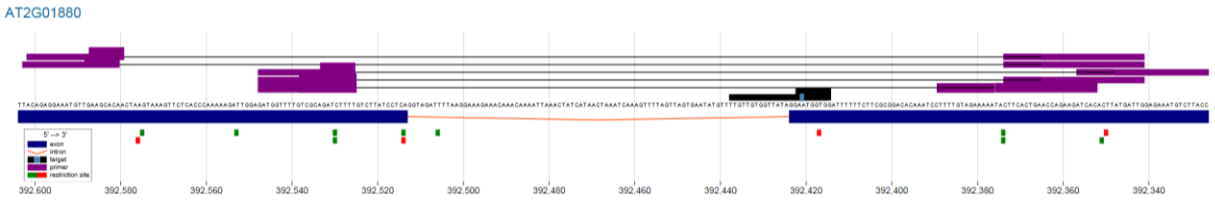
4 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
MboI:Sau3AI:DpnII:BfuCI cut GATC
DpnI cut GATC
AlwI cut GGATC
Nt.AlwI cut GGATC

T24 out of 30
<Previous Next>
Sequence: GAGAAAGAGGCTTCAAAGGAAGG
Oligo pair with 5' extension fwd: TAgGAGAAAGAGGCTTCAAAGGA rev: AAACTCCTTTGAAGCCTCTTTCT
Oligo pair with 5' substitution fwd: TAGgGAAAGAGGCTTCAAAGGA rev: AAACTCCTTTGAAGCCTCTTTC

Coordinates	strand	MM	target_seq	PAM	distance		gene name	gene id
chr2:393067-393089	+	0	GAGAAAGA[GGCTTCAAAGGA]	AGG	0	E	PAP7	AT2G01880
chr5:14671308-14671330	+	4	GTGCAAAA[GGCTTCAGAGGA]	AGG	0	E		AT5G37110
chr2:1809098-1809120	-	4	GTGCAAAA[GGCTTCAGAGGA]	AGG	0	E		AT2G05080
chr4:4152646-4152668	-	4	GTGCAAAA[GGCTTCAGAGGA]	AGG	0	E		AT4G07338
chr1:14066911-14066933	-	4	AGGAAAGA[GGCTAGAAAGGA]	TGG	0	E		AT1G37037

T25 out of 30

Spacer 2



Gene specific part of sgRNA
TGTTGTGGTTATAGGAATGGTGG exon 4
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr2:392527-392549	ATGGTTTTGTCGCAGATCTTTT	60.0	0	Chr2:392353-392377	TGTGATCTTCTGGTTCAGTGAAGT	60.2	0	0	196
2	Chr2:392527-392549	ATGGTTTTGTCGCAGATCTTTT	60.0	0	Chr2:392342-392366	CCAATCATAAGTGTGATCTTCTGG	59.9	0	0	207
3	Chr2:392527-392549	ATGGTTTTGTCGCAGATCTTTT	60.0	0	Chr2:392326-392349	TTGGTAAGACATTTCTCCAATCA	58.6	0	0	223
4	Chr2:392582-392604	ACAGAGGAAATGTTGAAGCACA	59.8	0	Chr2:392342-392366	CCAATCATAAGTGTGATCTTCTGG	59.9	0	0	262
5	Chr2:392581-392603	CAGAGGAAATGTTGAAGCACAA	60.3	0	Chr2:392342-392366	CCAATCATAAGTGTGATCTTCTGG				

Chr2:392418-392438:c	3	3	TGTTGTGGTTATAGGAATGG	TGGATTTTTT	-	exon

gRNA (Spacer was shown in upper-case):
5'-TGTTGTGGTTATAGGAATGGgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-TGTTGTGGTTATAGGAATGG-3'
5'-CCATTCCTATAACCACAACA-3'

GC content of Spacer sequence: 0.4

Potential Pol III terminator (TTTTT): null

0 from 149 REs recoginze Cas9 cut region (+7 to -13bp):

3.5.3 Cloning of PAP7 spacers (one, and two) into the binary vector/s pHEE401 and pHSE401

From section 3.4.5.1 gRNA1 (19-PAM): AGAAAGAGGCTTCAAAGGA *to be used in Forward primers*
gRNA2: GTTGTGGTTATAGGAATGG
gRNA2 reverse: CCATTCCTATAACCACAAC *to be used in reverse primers*

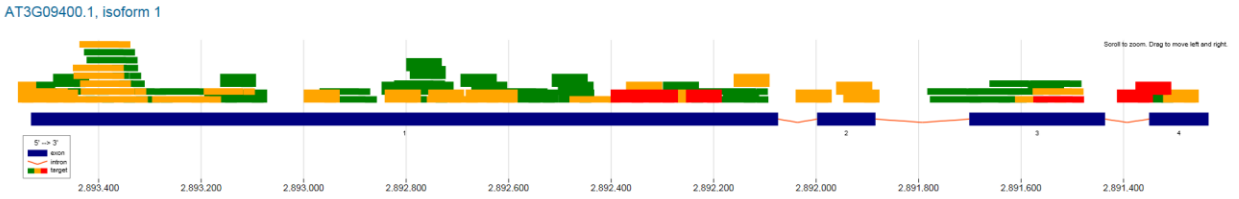
T1-BsF/PAP7 AATAATGGTCTCTATTGAGAAAGAGGCTTCAAAGGAGTT
T1-F0/PAP7 TGAGAAAGAGGCTTCAAAGGAGTTTTAGAGCTAGAAATAGC
T2-R0/PAP7 AACCCATTCCTATAACCACAACCAATCTCTTAGTCGACTCTAC
T2-BsR/PAP7 ATTATTGGTCTCTAAACCCATTCCTATAACCACAACC

3.6 Editing Protein phosphatase 2C (PPL2 and PLL3) using CRISPR CRISPR in Arabidopsis

3.6.1 PLL3 and PLL3?? Homology and peroxisomal identification

3.6.2 Predicting PLLs spacers

PLL3:



Ranking	Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
							0	1	2	3	
1	TGAAGTATTGGCGCTAACAGAGG	Chr3:2893281	1	+	48	0	0	0	0	0	0.73
2	GGACTGGTTCAAGAAAACCTCGG	Chr3:2893426	1	-	52	1	0	0	0	0	0.70
3	AGCAGGATCGCCTTCAGGGAAGG	Chr3:2891483	3	+	61	1	0	0	0	0	0.69
4	GGTCGGGTCTATTCGAACGCCGG	Chr3:2893131	1	-	65	0	0	0	0	0	0.66
5	TGACAGAGAAGTTGAGAGCGCGG	Chr3:2893257	1	+	57	0	0	0	0	0	0.65
6	AGAGGAGCCAAAGATACAATGGG	Chr3:2892785	1	-	48	0	0	0	0	0	0.64
7	CGTAGCAGAAAGAGTGGCCGAGG	Chr3:2893409	1	+	61	1	0	0	0	0	0.65
8	GGTTTCAGGGAAGAAGACCAAGG	Chr3:2893079	1	-	52	0	0	0	0	0	0.64
9	GAGCGTTGGAGATAGCCGAGCGG	Chr3:2892236	1	-	65	0	0	0	0	0	0.61
10	AGTCTATGGTGGTGTAGCGACGG	Chr3:2891604	3	+	57	1	0	0	0	0	0.60
11	GCCTCGTTCCATTTTGGCTGAGG	Chr3:2891685	3	+	57	1	0	0	0	0	0.60

Gene specific part of sgRNA
GTCGGAGGGGCGGTTCCGGCGGG
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr3:2893445-2893467	CGATACGTAACCGGAGTAGGAC	59.9	0	Chr3:2893280-2893302	TTGAAGTATTGGCGCTAACAGA	59.9	0	0	187
2	Chr3:2893402-2893424	ACTCTTTCTGCTACGTTCGTCC	60.0	0	Chr3:2893206-2893228	AGCGAATCTATTGGAACTCTCG	59.9	0	0	218
3	Chr3:2893448-2893470	GGTCGATACGTAACCGGAGTAG	59.9	0	Chr3:2893280-2893302	TTGAAGTATTGGCGCTAACAGA	59.9	0	0	190
4	Chr3:2893447-2893469	GTCGATACGTAACCGGAGTAGG	59.9	0	Chr3:2893280-2893302	TTGAAGTATTGGCGCTAACAGA	59.9	0	0	189
5	Chr3:2893445-2893467	CGATACGTAACCGGAGTAGGAC	59.9	0	Chr3:2893206-2893228	AGCGAATCTATTGGAACTCTCG	59.9	0	0	261

Class0.0 gRNA						
SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr3:2893351-2893371	NA	NA	GTCGGAGGGGCGGTTCCGGC	GGGAAAGAAG	+	exon

gRNA (Spacer was shown in upper-case):
5'-GTCGGAGGGGCGGTTCCGGCgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GTCGGAGGGGCGGTTCCGGC-3'
5'-GCCGGAACCGCCCCTCCGAC-3'

GC content of Spacer sequence: 0.8

Potential Pol III terminator (TTTTT): null

1 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
MspI:HpaII cut CCGG

Gene specific part of sgRNA
AGTCTATGGTGGTGTAGCGACGG
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr3:2891667-2891689	AGGCGCTTCTAGAGATGTTCAG	60.2	0	Chr3:2891401-2891423	ATCTTAAGTGCGTTTGCGTTTT	60.2	0	0	288
2	Chr3:2891667-2891689	AGGCGCTTCTAGAGATGTTCAG	60.2	0	Chr3:2891467-2891489	CTTGAATGAGATGTTGAGCAGG	59.9	0	0	222
3	Chr3:2891667-2891689	AGGCGCTTCTAGAGATGTTCAG	60.2	0	Chr3:2891500-2891522	GGAAGGCTGAGATGAATGAGTC	60.2	0	0	189
4	Chr3:2891667-2891689	AGGCGCTTCTAGAGATGTTCAG	60.2	0	Chr3:2891400-2891422	AATCTTAAGTGCGTTTGCGTTT	60.2	0	0	289
5	Chr3:2891667-2891689	AGGCGCTTCTAGAGATGTTCAG	60.2	0	Chr3:2891507-2891529	TGAGATGAATGAGTCAACCTCG	60.3	0	0	182

Chr3:2891603-2891623	3	5	AGTCTATGGTGGTGTAGCGA	CGGAGAGCAC	+	exon

gRNA (Spacer was shown in upper-case):
5'-AGTCTATGGTGGTGTAGCGAgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

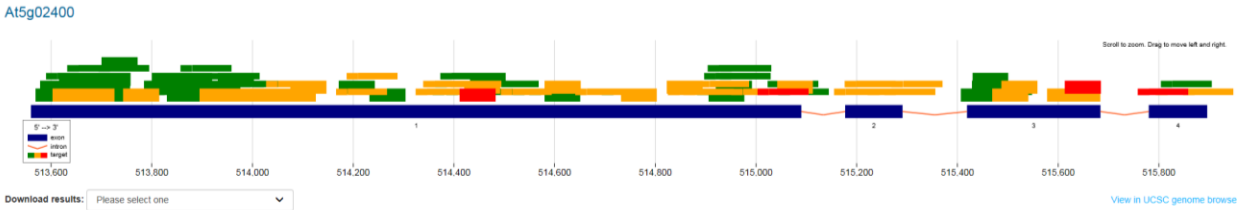
Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-AGTCTATGGTGGTGTAGCGA-3'
5'-TCGCTACACCACCATAGACT-3'

GC content of Spacer sequence: 0.5

Potential Pol III terminator (TTTTT): null

0 from 149 REs recoginze Cas9 cut region (+7 to -13bp):

PLL2



Ranking	Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
							0	1	2	3	
1	GCATCGTTCCATTTAGGCTGCGG	Chr5:515414	3	-	57	2	0	0	0	0	0.71
2	GGAGCTCTCGAATGCGGAGGCGG	Chr5:513856	1	-	70	1	0	0	0	0	0.67
3	GCGAAGGTAATACTACTCCGGGG	Chr5:513692	1	-	57	1	0	0	0	0	0.66
4	GTGGTTGTATCTGAAGACAACGG	Chr5:514359	1	+	48	0	0	0	0	0	0.65
5	CGGAGAGGAGACTCCGGCGAAGG	Chr5:513708	1	-	70	2	0	0	0	0	0.66
6	CGAGAGATACGTCGTAACGCCGG	Chr5:513623	1	-	61	1	0	0	0	0	0.62
7	GATCCTGAACATCTCTAAAAGGG	Chr5:515436	3	-	43	0	0	0	0	0	0.60
8	TGGATATGATCTGATCGGAGAGG	Chr5:513723	1	-	48	1	0	0	0	0	0.60
9	GATTGCCACGTGGAGCCACGCGG	Chr5:513912	1	-	70	2	0	0	0	0	0.61
10	GGTCAGCGCAAATCCTTCAACGG	Chr5:513779	1	+	57	0	0	0	0	0	0.58

Gene specific part of sgRNA
GGAGCTCTCGAATGCGGAGGCGG
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr5:513815-513837	TCTGTCATCGGATTCTGATTGT	59.6	0	Chr5:514039-514061	TTCTCTTTCTCCGTCTTCTTCG	60.1	0	0	246
2	Chr5:513740-513762	TATCCAAGAAACCACTTTCCGT	59.9	0	Chr5:513935-513957	CGACTCGTTAACAATTGGACCT	60.4	0	0	217
3	Chr5:513815-513837	TCTGTCATCGGATTCTGATTGT	59.6	0	Chr5:514034-514056	TTTCTCCGTCTTCTTCGTTGAT	60.2	0	0	241
4	Chr5:513815-513837	TCTGTCATCGGATTCTGATTGT	59.6	0	Chr5:514033-514055	TTCTCCGTCTTCTTCGTTGATT	60.2	0	0	240
5	Chr5:513719-513741	GTCTCCTCTCCGATCAGATCAT	59.7	0	Chr5:513935-513957	CGACTCGTTAACAATTGGACCT	60.4	0	0	238

Chr5:513858-513878:c	7	6	GGAGCTCTCGAATGCGGAGG	CGGAGACTGC	-	exon

gRNA (Spacer was shown in upper-case):
5'-GGAGCTCTCGAATGCGGAGGgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GGAGCTCTCGAATGCGGAGG-3'
5'-CCTCCGCATTCGAGAGCTCC-3'

GC content of Spacer sequence: 0.65

Potential Pol III terminator (TTTTT): null

1 from 149 REs recoginze Cas9 cut region (+7 to -13bp):
EciI cut GGCGGA

Gene specific part of sgRNA
GATTGAAAGCGGGTTGTATTCGG
There are no predicted off-targets for this guide

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr5:513883-513905	ATTTTGCCTCTTTACCACTCCA	60.0	0	Chr5:514108-514130	TTCGCGAATAGTGTTTTGAATG	60.1	0	0	247
2	Chr5:513883-513905	ATTTTGCCTCTTTACCACTCCA	60.0	0	Chr5:514149-514171	CACTCTTCTTCAAGCGAGGTTT	60.1	0	0	288
3	Chr5:513940-513962	CAATTGTTAACGAGTCGGGTCT	60.4	0	Chr5:514149-514171	CACTCTTCTTCAAGCGAGGTTT	60.1	0	0	231
4	Chr5:513883-513905	ATTTTGCCTCTTTACCACTCCA	60.0	0	Chr5:514107-514129	TCGCGAATAGTGTTTTGAATGT	59.7	0	0	246
5	Chr5:513878-513900	CGGTAATTTTGCCTCTTTACCA	60.3	0	Chr5:514108-514130	TTCGCGAATAGTGTTTTGAATG	60.1	0	0	252

SeqID	minMM_GG	minMM_AG	Spacer seq (5'->3')	PAM (5'->3')	strand	location
Chr5:514000-514020	NA	NA	GATTGAAAGCGGGTTGTATT	CGGGTCCGAT	+	exon

gRNA (Spacer was shown in upper-case):
5'-GATTGAAAGCGGGTTGTATTgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt-3'

Paired DNA oligos (without cloning adaptor) to construct gRNA :
5'-GATTGAAAGCGGGTTGTATT-3'
5'-AATACAACCCGCTTTCAATC-3'

GC content of Spacer sequence: 0.4

Potential Pol III terminator (TTTTT): null

0 from 149 REs recoginze Cas9 cut region (+7 to -13bp):

3.6.3 Cloning of spacers (one, and two) into the binary vector/s pHEE401 and pHSE401

PLL3

From section 3.6.2 gRNA1 (19-PAM): TCGGAGGGGCGGTTCCGGC *to be used in Forward primers*
gRNA2: GTCTATGGTGGTGTAGCGA
gRNA2 reverse: TCGCTACACCACCATAGAC *to be used in reverse primers*

T1-BsF/PLL3 AATAATGGTCTCTATTGTCGGAGGGGCGGTTCCGGCGTT
T1-F0/PLL3 TGTCGGAGGGGCGGTTCCGGCGTTTTAGAGCTAGAAATAGC
T2-R0/PLL3 AACTCGCTACACCACCATAGACCAATCTCTTAGTCGACTCTAC
T2-BsR/PLL3 ATTATTGGTCTCTAAACTCGCTACACCACCATAGACC

PLL2

From section 3.6.2 gRNA1 (19-PAM): GAGCTCTCGAATGCGGAGG *to be used in Forward primers*
gRNA2: ATTGAAAGCGGGTTGTATT
gRNA2 reverse: AATACAACCCGCTTTCAAT *to be used in reverse primers*

T1-BsF/PLL2 AATAATGGTCTCTATTGGAGCTCTCGAATGCGGAGGGTT
T1-F0/PLL2 TGGAGCTCTCGAATGCGGAGGGTTTTAGAGCTAGAAATAGC
T2-R0/PLL2 AACAATACAACCCGCTTTCAATCAATCTCTTAGTCGACTCTAC
T2-BsR/PLL2 ATTATTGGTCTCTAAACAATACAACCCGCTTTCAATC

References

Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz MM (2015) CRISPR/Cas9-mediated viral interference in plants. Genome Biol 16:238; doi:10.1186/s13059-015-0799-6

Brautigan DL (2013) Protein Ser/Thr phosphatases--the ugly ducklings of cell signalling. FEBS J 280:324-345; doi:10.1111/j.1742-4658.2012.08609.x

Brooks C, Nekrasov V, Lippman ZB, Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 166:1292-1297; doi:10.1104/pp.114.247577

Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. Genome Biol 16:232; doi:10.1186/s13059-015-0796-9

Chaparro-Garcia A, Kamoun S, Nekrasov V (2015) Boosting plant immunity with CRISPR/Cas. Genome Biol 16:254; doi:10.1186/s13059-015-0829-4

Ding Y, Li H, Chen LL, Xie K (2016) Recent Advances in Genome Editing Using CRISPR/Cas9. Front Plant Sci 7:703; doi:10.3389/fpls.2016.00703

Feng C, Yuan J, Wang R, Liu Y, Birchler JA, Han F (2016) Efficient Targeted Genome Modification in Maize Using CRISPR/Cas9 System. J Genet Genomics 43:37-43; doi:10.1016/j.jgg.2015.10.002

Gao J et al. (2015) CRISPR/Cas9-mediated targeted mutagenesis in Nicotiana tabacum. Plant Mol Biol 87:99-110; doi:10.1007/s11103-014-0263-0

Graham DB, Root DE (2015) Resources for the design of CRISPR gene editing experiments. Genome Biol 16:260; doi:10.1186/s13059-015-0823-x

Jiang Y, Xie M, Zhu Q, Ma X, Li X, Liu Y, Zhang Q (2013) One-step cloning of intron-containing hairpin RNA constructs for RNA interference via isothermal in vitro recombination system. Planta 238:325-330; doi:10.1007/s00425-013-1896-y

Kataya AR, Heidari B, Lillo C (2015a) Protein phosphatase 2A regulatory subunits affecting plant innate immunity, energy metabolism, and flowering time--joint functions among B'eta subfamily members. Plant Signal Behav 10:e1026024; doi:10.1080/15592324.2015.1026024

Kataya AR, Schei E, Lillo C (2015b) MAP kinase phosphatase 1 harbors a novel PTS1 and is targeted to peroxisomes following stress treatments. J Plant Physiol 179:12-20; doi:10.1016/j.jplph.2015.03.002

Kataya AR, Schei E, Lillo C (2016) Towards understanding peroxisomal phosphoregulation in Arabidopsis thaliana. Planta 243:699-717; doi:10.1007/s00425-015-2439-5

Khatodia S, Bhatotia K, Passricha N, Khurana SM, Tuteja N (2016) The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. Front Plant Sci 7:506; doi:10.3389/fpls.2016.00506

Li JF et al. (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol 31:688-691; doi:10.1038/nbt.2654

Lillo C, Kataya AR, Heidari B, Creighton MT, Nemie-Feyissa D, Ginbot Z, Jonassen EM (2014) Protein phosphatases PP2A, PP4 and PP6: mediators and regulators in development and responses to environmental cues. Plant Cell Environ 37:2631-2648; doi:10.1111/pce.12364

Liu W, Zhu X, Lei M, Xia Q, Botella JR, Zhu J-K, Mao Y (2015) A detailed procedure for CRISPR/Cas9-mediated gene editing in Arabidopsis thaliana. Science Bulletin 60:1332-1347; doi:10.1007/s11434-015-0848-2

Pan C et al. (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. Sci Rep 6:24765; doi:10.1038/srep24765

Shen B et al. (2014) Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods 11:399-402; doi:10.1038/nmeth.2857

Uhrig RG, Labandera AM, Moorhead GB (2013) Arabidopsis PPP family of serine/threonine protein phosphatases: many targets but few engines. Trends Plant Sci 18:505-513; doi:10.1016/j.tplants.2013.05.004

Woo JW et al. (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33:1162-1164; doi:10.1038/nbt.3389

Xing H-L et al. (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biology 14:1-12; doi:10.1186/s12870-014-0327-y

Zuris JA et al. (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol 33:73-80; doi:10.1038/nbt.3081