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High-resolution mapping of *zym*, a recessive gene for *Zucchini yellow mosaic virus* resistance in cucumber

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Abstract

Key message **Using a high-resolution mapping approach, we identified a candidate gene for ZYMV resistance in cucumber. Our findings should assist the development of high-versatility molecular markers for MAS for ZYMV resistance.**

Abstract *Zucchini yellow mosaic virus* (ZYMV) causes significant disease, which leads to fruit yield loss in cucurbit crops. Since ZYMV resistance is often inherited recessively in cucumber, marker-assisted selection (MAS) is a useful tool for the development of resistant cucumber cultivars. Using 128 families of an $F_{2:3}$ population derived from a cross between susceptible 'CS-PMR1' and resistant 'A192-18' cucumber inbred lines, we confirmed that ZYMV resistance is conferred by a single recessive locus:
$zym^{A192-18}$. We constructed a cucumber genetic linkage map that included 125 simple sequence repeat (SSR) markers segregating into 7 linkage groups (chromosomes). The $zym^{A192-18}$ locus was mapped to chromosome 6, at genetic distances of 0.9 and 1.3 cM from two closely linked SSR markers. For high-resolution genetic mapping, we identified new molecular markers cosegregating with the $zym^{A192-18}$ locus; using cucumber genomic and molecular marker resources and screening an F_2 population of 2,429 plants, we narrowed down the $zym^{A192-18}$ locus to a <50-kb genomic region flanked by two SSR markers, which included six candidate genes. Sequence analysis of the candidate genes' coding regions revealed that the vacuolar protein sorting-associated protein 4-like (VPS4-like) gene had two SNPs between the parental lines. Based on SNPs of the VPS-4-like gene, we developed $zym^{A192-18}$-linked DNA markers and found that genotypes associated with these markers were correlated with the ZYMV resistance phenotype in 48 cucumber inbred lines. According to our data, the gene encoding VPS4-like protein is a candidate for the $zym^{A192-18}$ locus. These results may be valuable for MAS for ZYMV resistance in cucumber.

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Introduction

Zucchini yellow mosaic virus (ZYMV), an aphid-borne potyvirus, causes significant damage to cucurbit crop production worldwide (Gal-On 2007). Cucurbit plantings are particularly vulnerable from summer through early autumn, when increased aphid vector populations promote cucurbit virus epidemics that eventually lead to declines in fruit yield (Kosaka et al. 2006). Since no chemical substances have yet been found to effectively control the virus in infected plants, breeding for ZYMV resistance is the most

desirable approach for virus disease control. In cucumber (*Cucumis sativus* L.), sources of ZYMV resistance have been identified. Inheritance of this trait has been well characterized in inbred lines derived from the Taiwanese cultivar 'Taichung Mou Gua' (TMG-1) and the Dutch hybrid 'Dina' (Dina-1), where resistance is due to a recessive allele at a single locus: zym^{TMG-1} and zym^{Dina-1}, respectively (Kabelka et al. 1997). ZYMV resistance in a Japanese-type cucumber inbred line has also been shown to be controlled by a single recessive allele (Cardoso et al. 2010). These findings suggest that ZYMV resistance in cucumber is often inherited recessively. To introduce a recessive allele conferring ZYMV resistance into a breeding line, plants must be self-pollinated after generations of backcrossing and then inoculated with the virus to examine resistance, lengthening the time required to develop resistant plants. This time can be shortened using marker-assisted selection (MAS). Several DNA markers linked to the recessive ZYMV resistance gene (*zym*) have been developed (Park et al. 2000, 2004). Since these markers may not be polymorphic between some ZYMV-resistant and susceptible plants (Park et al. 2004), distinct molecular markers more tightly linked to the *zym* locus are needed for MAS.

The whole genomes of three cucumber inbred lines—the Northern Chinese fresh market-type '9930' (Huang et al. 2009), the North American pickling type 'Gy14' (Cavagnaro et al. 2010), and the European inbred line 'B10' (Wóycicki et al. 2011)—have recently been sequenced, and cucumber genome databases containing these sequence data, such as the International Cucurbit Genomics Initiative (ICuGI) and Phytozome v9.1 (Goodstein et al. 2012), have been made publicly accessible. In addition, many genome-wide cucumber simple sequence repeat (SSR) markers derived from genomic or expressed sequence tag libraries and whole-genome sequence data have been developed (Kong et al. 2006; Fukino et al. 2008; Watcharawongpaiboon and Chunwongse 2008; Ren et al. 2009; Cavagnaro et al. 2010; Guo et al. 2010). These genomic and molecular marker resources have been successfully used for high-density genetic map construction (Ren et al. 2009; Miao et al. 2011; Fukino et al. 2013) and for identification or cloning of candidate genes for horticulturally important traits (Li et al. 2009, 2011; Kang et al. 2011).

In this study, we initially constructed a framework genetic linkage map to identify SSR markers linked to the *zym* locus based on reference cucumber genetic maps. To develop new molecular markers, we then carried out high-resolution genetic mapping and identified the most likely candidate gene for the *zym* locus using the cucumber genome and molecular marker resources. Our findings can be used in MAS aimed at cucumber breeding for ZYMV resistance, and for map-based cloning of the identified cucumber ZYMV resistance gene.

Materials and methods

Plant materials

The Japanese fresh market-type ZYMV-resistant inbred line 'A192-18', derived from self-pollinated progeny of the F_1 hybrid 'Sagamihanjiro fushinari (SF) × G22' (Svoboda et al. 2013), and a susceptible weedy cucumber of Indian origin, 'CS-PMR1' (Fukino et al. 2013), were used as parents. To determine the inheritance pattern of the ZMVY resistance trait, F_1, F_2, and backcross populations from the cross between A192-18 and CS-PMR1 were generated in a greenhouse. A total of 128 F_3 families were obtained by self-pollination of each F_2 line ($F_{2:3}$ population) for genome framework mapping. At least 25 plants from the respective F_3 families were used for genetic analysis of ZYMV resistance. For high-resolution mapping, an additional 2,301 F_2 plants from the same cross were used.

Virus inoculation and detection procedures

ZYMV Z5-1 (Kosaka and Fukunishi 1997) was maintained on cucumber inbred line 'SF'. Infected leaves were collected about 3 weeks after inoculation and stored at −80 °C before being used as an inoculum source. Viral inoculum was prepared by grinding the infected leaves (1:10 w/v) in 0.05 M phosphate buffer (pH 7.2) with a mortar and pestle. Carborundum-dusted cotyledons of 7- to 8-day-old seedlings were mechanically inoculated by rubbing them with cotton swabs dipped in the inoculum. The inoculated plants were grown in a growth chamber (KCLP-1400CCFL-CT; Nippon Medical & Chemical Instruments, Osaka, Japan) at 25 °C with a 16-h light/8-h dark photoperiod. Two weeks later, disease resistance of each plant was scored using a five-step disease rating scale: 0 (no symptoms), 1 (slight mosaic limited to lower leaves), 2 (pronounced mosaic on lower leaves and slight mosaic on upper leaves), 3 (moderate mosaic on upper leaves), or 4 (severe mosaic on all leaves). In the 128 $F_{2:3}$ families, disease resistance was evaluated using a disease index (DI) following Zhang et al. (2010), where DI = $\Sigma[(s \times n)/(S \times N)] \times 100$, with s = disease rating scale, n = number of plants with each disease rating, N = total number of plants, and S = highest disease rating scale. ZYMV resistance for each $F_{2:3}$ family was evaluated based on its DI value: families with DI ≤ 15 were considered resistant, those with $15 < DI < 85$ were considered intermediate, and those with DI ≥ 85 were considered susceptible. Backcross plants were evaluated based on disease ratings of individual plants, with resistant (R) = a disease rating of 0 or 1, intermediate (I) = 2, and susceptible (S) = 3 or 4.

Virus detection in young developed leaves was performed using a double-antibody sandwich enzyme-linked

immunosorbent assay (DAS-ELISA) or by reverse transcription-polymerase chain reaction (RT-PCR) (Kosaka et al. 2006). DAS-ELISA was carried out with a ZYMV-specific antiserum (Japan Plant Protection Association, Ibaraki, Japan). Absorbance was measured at 405 nm using an iMark Microplate Absorbance Reader (BIO-RAD Laboratories, Hercules, CA, USA). Samples were considered positive if measured absorbance was more than twice that of healthy plants. Data were expressed as mean absorbance plus standard deviation of results from triplicate experiments. For RT-PCR, total RNA was extracted with Sepasol RNAI Super reagent (Nacalai Tesque, Kyoto, Japan). After treatment with RNase-free DNase (Wako, Osaka, Japan), total RNA (1 μg) was reverse-transcribed into cDNA using a cDNA synthesis kit (Reverse Transcription System; Promega, Fitchburg, WI, USA) according to the manufacturer's protocol. An aliquot (1 μl) of 10-fold-diluted cDNA was subjected to PCR with Taq DNA polymerase (Takara, Kyoto, Japan) in the presence of 0.2 μM of each respective ZYMV-specific primer (forward: 5′-TCTCATCAGCA ATTCGCCTC-3′ and reverse: 5′-AGAAACATTGCTAAG GGCTG-3′). PCR amplification was performed on a GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, USA) using the following protocol: 94 °C for 1 min; 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s; and 72 °C for 3 min. PCR products (393 bp; Shimomoto and Takeuchi 2006) were separated by electrophoresis on a 1.5 % (w/v) agarose gel. The gels were stained with ethidium bromide (0.5 μg/ml); bands were visualized under UV light and photographed with a digital gel-documentation system (AE-6905N; ATTO, Tokyo, Japan).

Framework and high-resolution genetic mapping

To construct a whole-genome framework map of the $F_{2:3}$ population, a cucumber genetic map with SSR markers distributed across all seven chromosomes (Fukino et al. 2013) was used as reference during initial screening for polymorphic SSR markers in parental lines. Additional SSR markers (Ren et al. 2009) were used when clearly polymorphic SSR markers could not be obtained from the reference map. For high-resolution genetic mapping, an additional larger F_2 population (2,301 plants) was grown in a greenhouse. Young leaf samples were collected for DNA extraction to identify recombinants defined by SSR markers flanking the *zym* locus in the $F_{2:3}$ population. Recombinant F_2 plants were planted to obtain respective F_3 families. At least 25 plants from the respective F_3 families were evaluated for ZYMV resistance using a ZYMV inoculation test. ZYMV resistance in respective F_3 families was scored using a DI as described above. The cucumber genome database Phytozome v9.1, based on the whole-genome sequence of Gy14 (Cavagnaro et al. 2010; http://www.phytozome.net/

cucumber.php), was used to identify gene scaffolds containing SSR markers linked to the *zym* locus in the $F_{2:3}$ population. New parental polymorphic SSR markers were subsequently uncovered based on SSR markers on the gene scaffold (Cavagnaro et al. 2010). These new markers were applied to all recombinant plants in the large F_2 population to construct a high-resolution map around the *zym* locus, and to narrow down the *zym* locus flanking region. Physical distances between *zym* locus flanking SSR markers were obtained using two cucumber genome databases: Phytozome v9.1, based on the whole-genome sequence of Gy14 (Goodstein et al. 2012), and the Cucurbit Genomics Database. The latter database, derived from the whole-genome sequence of 9930 (Huang et al. 2009), is maintained by the ICuGI (http://www.icugi.org/).

DNA sequencing of candidate genes

Potential candidate genes for the *zym* locus were identified by examining two databases (Phytozome v9.1 and ICuGI); their coding regions were sequenced to find single-nucleotide polymorphisms (SNPs) between the parental lines using sequencing primers designed with the Primer 3.0 program (Rozen and Skaletsky 2000). Neighboring primer pairs were designed to amplify PCR products with at least 100-bp overlap with the preceding fragment. PCR products were resolved by electrophoresis on a 1.5 % agarose gel. Excised bands were purified with a HighPure PCR Product Purification kit (Roche Diagnostics, Basel, Switzerland), and cloned into a pGEM-T EASY vector (Promega). The cloned fragments were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing kit on an ABI PRISM 3130 sequencer (Life Technologies). Functional annotations of candidate genes were retrieved from their database entries at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih. gov).

Validation of molecular markers linked to the *zym* locus

To validate the *zym* locus-linked molecular markers, we used 48 cucumber inbred lines originating from Japan, southern Asia, northern and southern China, the Middle East, Europe (greenhouse type), and North America (processing and slicing types). The resistance phenotype of each line (at least 25 plants per line) was identified using a ZYMV inoculation test.

Molecular marker development and analysis

Genomic DNA was isolated from young leaves using the CTAB method (Murray and Thompson 1980) and used for marker analysis. PCRs were carried out in 10-μl reaction

volumes containing 10–20 ng genomic DNA, 0.2 µM of each primer, 100 µM dNTPs, 1× reaction buffer, and 0.5 U Taq DNA polymerase (Takara). Amplifications were performed on a GeneAmp PCR System 9700 (Life Technologies) according to the following protocol: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; and 72 °C for 3 min. Amplified products were separated using an electrophoresis system (NA-1040; NIHON EIDO, Tokyo Japan) on 5 % (stacking) and 13 % (resolving) non-denaturing polyacrylamide gels in 1× Tris–glycine buffer according to Xu et al. (2009). After migration at 120 V for 2 h, gels were stained with ethidium bromide (0.5 µg/ml), and the bands were visualized and photographed as described above.

The cucumber DNA cloned genomic region YCZ-5 (GenBank accession numbers AY254907 and AY254908), which covers DNA markers linked to the zym^{TMG-1} locus (Park et al. 2004), was sequenced from each parental line to identify SNPs and insertions/deletions (Indels). This region was monomorphic between our parental lines; inverse PCR (IPCR; Triglia et al. 1988) or thermal asymmetric interlaced-PCR (TAIL-PCR; Liu and Whittier 1995) was therefore carried out to obtain YCZ-5 5′- and 3′-flanking sequences.

Identified SNPs of candidate genes were used to develop cleaved amplified polymorphic sequence markers (CAPS; Konieczny and Ausubel 1993) or derived CAPS (dCAPS; Neff et al. 1998) using appropriate restriction enzymes (RE) (Table S1). CAPS and dCAPS primers were designed using Primer 3.0 (Rozen and Skaletsky 2000) and dCAPS Finder 2.0 (Neff et al. 2002), respectively. After PCR with specific primers, the resulting amplicons were mixed with 1 U of RE in buffer (New England BioLabs, Ipswich, MA, USA) and incubated for 3 h at temperatures optimized for each RE. Digestion products were separated by electrophoresis on a 2–3 % (w/v) agarose gel, and bands were visualized and photographed as described above. Information on the *zym* locus-linked SSR markers and newly developed molecular markers, as well as primer sequences, is provided in Supplemental Table 1.

Semi-quantitative RT-PCR

To validate expression of candidate genes, young developed leaves (5 days after inoculation with ZYMV) from both parents were collected and flash frozen in liquid nitrogen. Total RNA extraction and reverse transcription to cDNA were performed as described above. PCR amplification was carried out using the following reaction conditions: 94 °C for 2 min, followed by 23–28 cycles of 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 60 s, with a final step of 72 °C for 3 min. Actin (GenBank accession number AB010922) was used as an internal standard. PCR products

were separated by electrophoresis on a 1.5 % (w/v) agarose gel. Gels were stained with ethidium bromide (0.5 µg/ml) and bands visualized and photographed as described above. All experiments were performed with three technical replicates. Candidate gene and actin primers were designed using Primer 3.0 (Rozen and Skaletsky 2000); primer sequences are listed in Supplemental Table 3.

Data analysis

Phenotype and molecular marker data were combined for linkage analysis using Mapmaker v. 3.0, with a logarithm of odds (LOD) threshold of 3.0 (Lander et al. 1987). Recombination percentages were converted to genetic distances using the Kosambi mapping function (Kosambi 1944). Maps were drawn with MapChart v. 2.1 (Voorrips 2002).

Results

Genetic analysis of ZYMV resistance in A192-18

Approximately 14 days post-inoculation, the susceptible line 'CS-PMR1' and F_1 plants showed mosaic symptoms, whereas the resistant line 'A192-18' appeared symptom-free (Fig. 1a). DAS-ELISA values for upper leaves of A192-18 plants were significantly lower than for CS-PMR1 and F_1 plants (A192-18: 0.006 ± 0.001; CS-PMR1: 1.283 ± 0.147; F_1: 1.240 ± 0.128; Fig. 1b). RT-PCR analysis revealed the presence of ZYMV in CS-PMR1 and F_1 plants, but not in A192-18 (Fig. 1c). Based on ZYMV inoculation tests of $F_{2:3}$ families, F_2 parents exhibited a resistant (*R*): intermediate (*I*): susceptible (*S*) ratio of approximately 1:2:1, whereas the $F_1 \times$ A192-18 backcross population segregated into a *R*:*S* ratio close to 1:1 and the $F_1 \times$ CS-PMR1 backcross population was entirely susceptible (Table 1). These results confirmed that ZYMV resistance in A192-18 is conferred by a single recessive gene locus (designated as $zym^{A192-18}$).

Mapping of the $zym^{A192-18}$ locus and identification of linked molecular markers

Of 311 SSR markers on 7 linkage groups (LG1–LG7) or chromosomes (Chr1–Chr7) (Ren et al. 2009; Fukino et al. 2013), 125 (40.2 %) were clearly polymorphic between A192-18 and CS-PMR1. Our cucumber framework genetic map constructed with seven linkage groups (LG1–LG7) had a total length of 677.6 cM, with an average marker interval of 6.27 cM. Most of the common SSR markers in this framework genetic map were obtained from the integrated genetic and cytogenetic cucumber genomic map (Ren et al. 2009). In line with previous reports (Li et al. 2011; Fukino et al. 2013), markers on our linkage map (LG1–LG7) could

Fig. 1 Susceptibility of cucumber lines A192-18, CS-PMR1, and their F_1 progeny to ZYMV Z5-1. **a** Seedlings 14 days after inoculation. **b** Mean absorbance using DAS-ELISA analysis. *Error bars* represent standard deviation from triplicate experiments. Data sets marked with an *asterisk* are significantly different from F_1 and CS-PMR1 as assessed by Student's *t* test; ***P* < 0.001. **c** RT-PCR analysis of young developed leaves of A192-18 (*lane 1*), F_1 (*lane 2*), and CS-PMR1 (*lane 3*). M, 100-bp DNA ladder
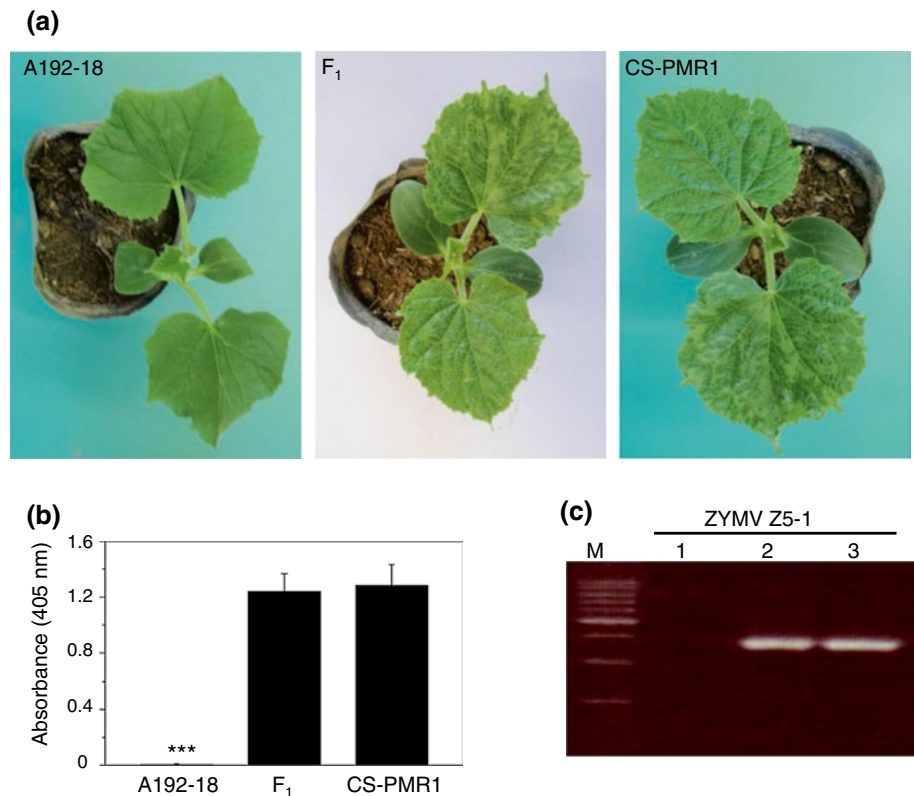

Table 1 Segregation analysis of ZYMV resistance in the $F_{2:3}$ and BC_1 populations derived from cucumber lines A192-18 and CS-PMR1

Population	Observed no. of plants						
	Plants tested	R	I	S	Expected ratio	χ^{2a}	P
$F_{2:3}$	128	32	62	34	1:2:1	0.91	0.63
BC_1 (F_1 × A192-18)	106	58	–	48	1:1	0.33	0.57
BC_1 (F_1 × CS-PMR1)	112	0	–	112	0:1	–	–

R resistant, I intermediate, S susceptible

[a] $\chi^2 (0.05,1) = 3.84$, $\chi^2 (0.05,2) = 5.99$

be assigned to seven chromosomes (Chr1–Chr7; Fig. 2). The $zym^{A192-18}$ locus was located on Chr6, flanked by markers SSR02385 and CSN227 at a distance of 9.9 and 3.0 cM, respectively. The SSR marker CU2345 cosegregated with the $zym^{A192-18}$ locus (Figs. 2, 3a).

Forty SSR markers were found in the flanking regions of the $zym^{A192-18}$ locus between SSR02385 and SSR00842 (Ren et al. 2009). In our study, five of them were clearly polymorphic between parental lines; these markers were used for mapping around the $zym^{A192-18}$ locus in our framework genetic map. SSR18780 and SSR07248 were located on one side of the $zym^{A192-18}$ locus, whereas SSR13884, SSR31399, and SSR17909 were located on the other. Genetic distances between the $zym^{A192-18}$ locus and the closest flanking markers (SSR07248 and SSR13884) were 0.9 and 1.3 cM, respectively (Fig. 3b). We sequenced the YCZ-5 region, which covers DNA markers linked to the zym^{TMG-1} locus (Park et al. 2004), from each parent, but no SNPs were uncovered between the parental lines in that region. We consequently generated 5′- and 3′-flanking sequences of the YCZ-5 region by IPCR or TAIL-PCR (primer sequences are listed in Table S2). An Indel between the parents was identified in the 3′-flanking sequence, approximately 9 kb away from the YCZ-5 region. We then developed an Indel marker, T1, and found that it also cosegregated with the $zym^{A192-18}$ locus in the framework genetic map (Fig. 3b).

High-resolution mapping of the $zym^{A192-18}$ locus

The $zym^{A192-18}$ locus-linked SSR markers—SSR07248, SSR13884, and CU2345—were physically located on the
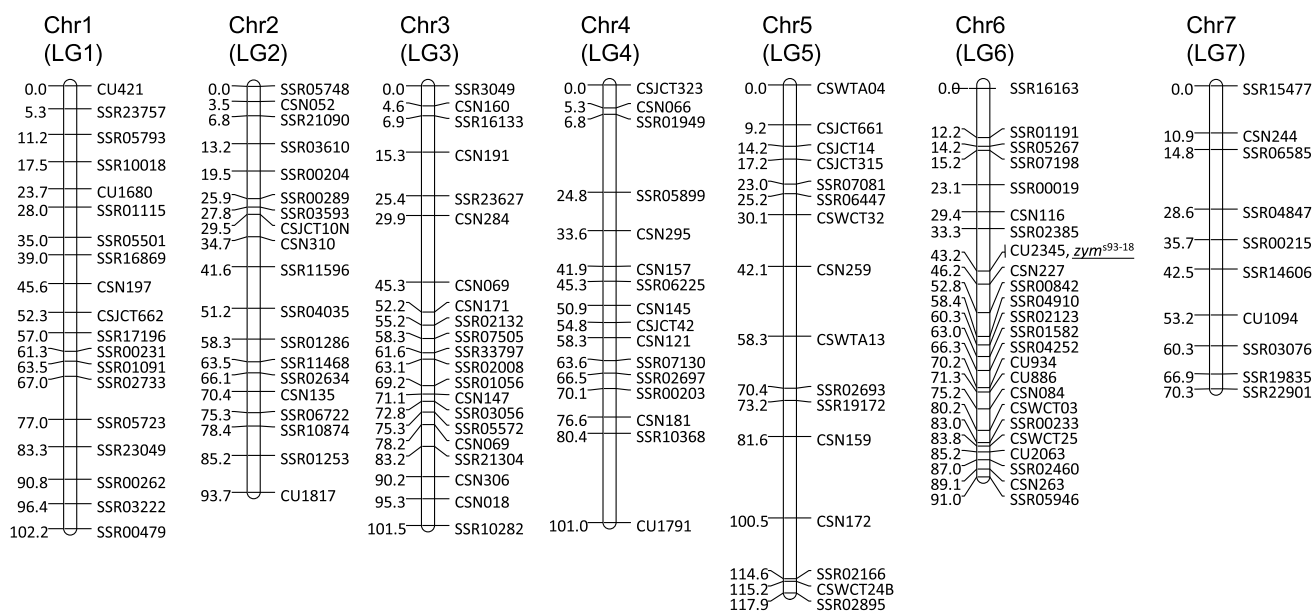
Chr1 (LG1)

cM	Marker
0.0	CU421
5.3	SSR23757
11.2	SSR05793
17.5	SSR10018
23.7	CU1680
28.0	SSR01115
35.0	SSR05501
39.0	SSR16869
45.6	CSN197
52.3	CSJCT662
57.0	SSR17196
61.3	SSR00231
63.5	SSR01091
67.0	SSR02733
77.0	SSR05723
83.3	SSR23049
90.8	SSR00262
96.4	SSR03222
102.2	SSR00479

Chr2 (LG2)

cM	Marker
0.0	SSR05748
3.5	CSN052
6.8	SSR21090
13.2	SSR03610
19.5	SSR00204
25.9	SSR00289
27.8	SSR03593
29.5	CSJCT10N
34.7	CSN310
41.6	SSR11596
51.2	SSR04035
58.3	SSR01286
63.5	SSR11468
66.1	SSR02634
70.4	CSN135
75.3	SSR06722
78.4	SSR10874
85.2	SSR01253
93.7	CU1817

Chr3 (LG3)

cM	Marker
0.0	SSR3049
4.6	CSN160
6.9	SSR16133
15.3	CSN191
25.4	SSR23627
29.9	CSN284
45.3	CSN069
52.2	CSN171
55.2	SSR02132
58.3	SSR07505
61.6	SSR33797
63.1	SSR02008
69.2	SSR01056
71.1	CSN147
72.8	SSR03056
75.3	SSR05572
78.2	CSN069
83.2	SSR21304
90.2	CSN306
95.3	CSN018
101.5	SSR10282

Chr4 (LG4)

cM	Marker
0.0	CSJCT323
5.3	CSN066
6.8	SSR01949
24.8	SSR05899
33.6	CSN295
41.9	CSN157
45.3	SSR06225
50.9	CSN145
54.8	CSJCT42
58.3	CSN121
63.6	SSR07130
66.5	SSR02697
70.1	SSR00203
76.6	CSN181
80.4	SSR10368
101.0	CU1791

Chr5 (LG5)

cM	Marker
0.0	CSWTA04
9.2	CSJCT661
14.2	CSJCT14
17.2	CSJCT315
23.0	SSR07081
25.2	SSR06447
30.1	CSWCT32
42.1	CSN259
58.3	CSWTA13
70.4	SSR02693
73.2	SSR19172
81.6	CSN159
100.5	CSN172
114.6	SSR02166
115.2	CSWCT24B
117.9	SSR02895

Chr6 (LG6)

cM	Marker
0.0	SSR16163
12.2	SSR01191
14.2	SSR05267
15.2	SSR07198
23.1	SSR00019
29.4	CSN116
33.3	SSR02385
43.2	CU2345, zym^{s93-18}
46.2	CSN227
52.8	SSR00842
58.4	SSR04910
60.3	SSR02123
63.0	SSR01582
66.3	SSR04252
70.2	CU934
71.3	CU886
75.2	CSN084
80.2	CSWCT03
83.0	SSR00233
83.8	CSWCT25
85.2	CU2063
87.0	SSR02460
89.1	CSN263
91.0	SSR05946

Chr7 (LG7)

cM	Marker
0.0	SSR15477
10.9	CSN244
14.8	SSR06585
28.6	SSR04847
35.7	SSR00215
42.5	SSR14606
53.2	CU1094
60.3	SSR03076
66.9	SSR19835
70.3	SSR22901

Fig. 2 Framework genetic map based on 128 F$_{2:3}$ families derived from the cross A192-18 × CS-PMR1. The ZYMV resistance gene locus ($zym^{A192-18}$, *underlined*) mapped onto Chr6. Distances are shown in centimorgans (cM). *LG* linkage group, *Chr* chromosome

same scaffold (scaffold03904) of Gy14 (Phytozome v9.1); the physical distance between SSR07248 and SSR13884 was approximately 295 kb on scaffold03904 and 288 kb on Chr6 (ICuGI). According to genome-wide SSR marker resources (Cavagnaro et al. 2010), 144 SSR markers are present on scaffold03904 between SSR07248 and SSR13884. Based on their physical positions, 95 of these markers were chosen for polymorphism screening between the parents. As a result, 10 polymorphic SSR markers were newly identified that cosegregated with the $zym^{A192-18}$ locus in our framework genetic map.

Of the 2,429 F$_2$ plants (128 from F$_{2:3}$ families, and 2,301 from the additional larger F$_2$ population), 124 showed evidence of recombination between SSR07248 and SSR13884. These recombinant plants were used for high-resolution genetic mapping with the new SSR markers. The high-resolution map of the $zym^{A192-18}$ locus and the number of recombinants in each chromosomal segment (defined on the basis of adjacent markers) are shown in Fig. 3c. The positions of all markers were consistent with those on scaffold03904 of Gy14 and on Chr6 of 9930. SSR markers UW080856 and UW080841 flanked the $zym^{A192-18}$ locus, whereas UW080853 cosegregated with it (Fig. 3c). The physical distance between UW080856 and UW080841 was approximately 47 kb in Gy14 (scaffold03904; Phytozome v9.1) and 46 kb in 9930 (Chr6; ICuGI).

Candidate genes in the $zym^{A192-18}$ region

Phytozome v9.1 identified seven putative genes between UW080856 and UW080841, six of which were also identified by ICuGI. No genes were found in this region encoding either translation initiation factors typical of recessive resistance genes against plant viruses (Maule et al. 2007) or nucleotide binding site type and leucine-rich repeat proteins of the large family of plant disease resistance genes (Marone et al. 2013). Positions and predicted functions of candidate genes are summarized in Fig. 3d and Table 2. We carried out semi-quantitative RT-PCR analysis and sequenced the coding regions of the six genes identified by ICuGI. As shown in Fig. 4, gene expressions of the six candidate genes were not significantly different between A192-18 and CS-PMR1. In addition, their expressions were not enhanced or reduced by ZYMV inoculation (data not shown). When these gene regions were sequenced, no SNPs causing amino acid substitutions were detected except for the vacuolar protein sorting-associated protein 4 (VPS4)-like gene (gene no. 1 in Table 2). This VPS4-like gene featured two SNPs (T86C and G99A) leading to amino acid substitutions (Phe29Ser and Met33Ile) (Fig. 5b), suggesting that it might be the $zym^{A192-18}$ candidate gene. In this gene, eight exons were predicted based on the two cucumber genome databases; its deduced protein sequence contained three functional domains: a microtubule interaction and transport (MIT) domain, an ATPase family associated with various cellular activities (AAA), and a Vps4 C-terminal oligomerization (Vps4 C) domain. The SNPs were located in the first exon, which encodes the MIT domain (Fig. 5a). We also analyzed nucleotide sequences of the VPS4 coding region of ZYMV-resistant 'G22' and susceptible 'SF' Japanese-type inbred lines (Svoboda et al. 2013). Sequence alignment between G22
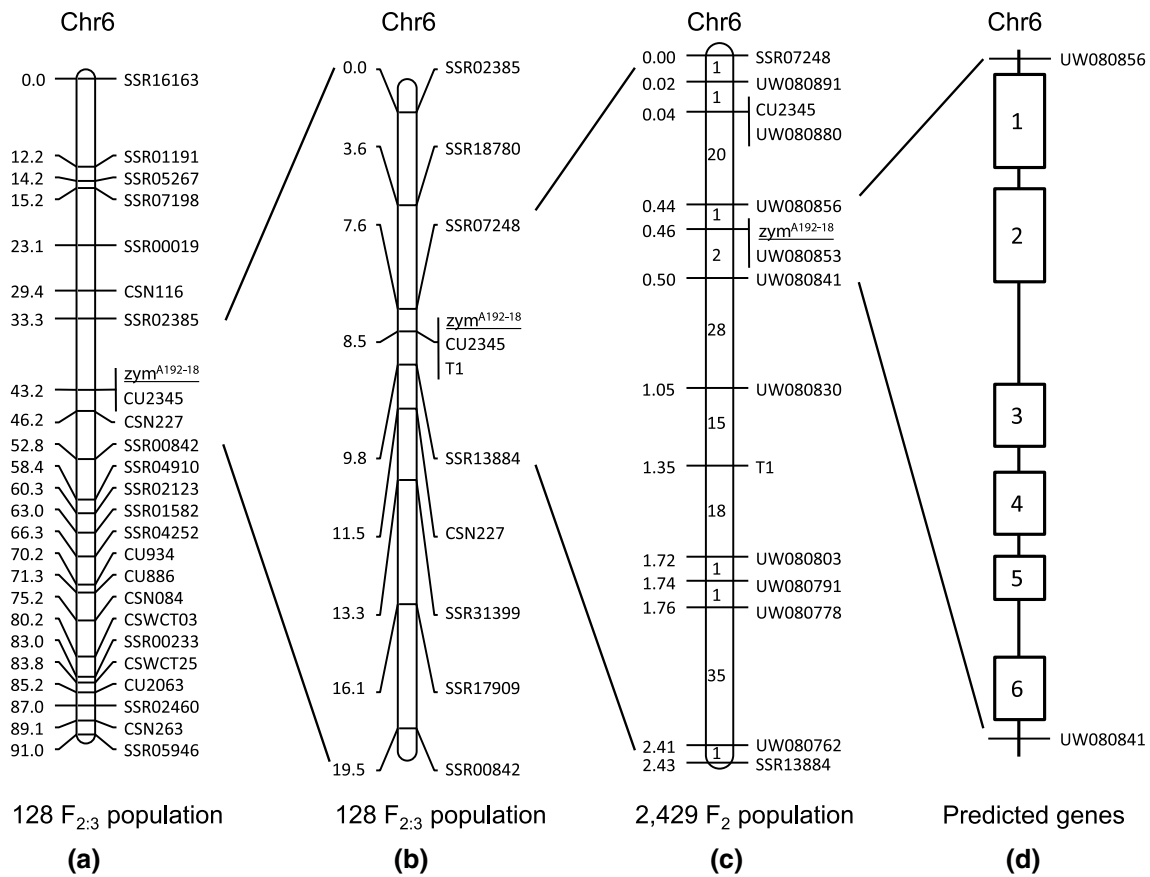
Fig. 3 High-resolution genetic map and physical map of the $zym^{A192-18}$ locus. **a** Mapping of the $zym^{A192-18}$ gene on Chr6 in the F$_{2:3}$ population derived from the cross A192-18 × CS-PMR1. **b** A local linkage map of the $zym^{A192-18}$ flanking region (between SSR02385 and SSR00842). **c** High-resolution genetic map including the $zym^{A192-18}$ locus, based on 2,429 F$_2$ plants. *Numbers between pairs of marker loci* indicate the number of recombination events in these regions. **d** The genomic region between UW080856 and UW080841 (≈50 kb), in which six genes (*white boxes*) were predicted; gene numbers correspond to those in Table 2

Table 2 Predicted genes between the SSR markers UW080856 and UW080841 flanking the $zym^{A192-18}$ locus on Chromosome 6

Gene no.	Predicted genes (ID)		CDS start position in Chr 6	Predicted gene function	Best Arabidopsis hit (Blast)	E value
	Phytozome v9.1	ICuGI				
1	Cucsa.385040	Csa6M152960.1	10850899	VPS4/Vacuolar protein sorting-associated protein	AT2G27600.1	4.00E−232
2	Cucsa.385030	Csa6M153460.1	10857142	APL1 (ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1)	AT5G19220.1	5.00E−233
3	Cucsa.385020	Csa6M153470.1	10867566	RHF1A (RING-H2 GROUP F1A)/ ubiquitin-protein ligase	AT4G14220.1	3.00E−71
4	Cucsa.385010	Csa6M153480.1	10875150	PRXIIF (PEROXIREDOXIN IIF)/ peroxidase	AT3G06050.1	1.00E−89
5	Cucsa.385000	Csa6M153490.1	10877050	Unnamed protein product	No hits found	–
6	Cucsa.384990	Csa6M153500.1	10882064	Unknown function (PC-Esterase domain)	AT5G19160.1	8.00E−185

ID identification number given by Phytozome v9.1 and ICuGI, *CDS* coding DNA sequence
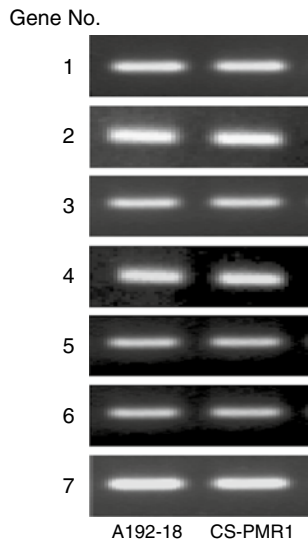
Gene No.

Fig. 4 Semi-quantitative RT-PCR analysis of six candidate genes in A192-18 and CS-PMR1. Candidate gene numbers (gene nos. 1–6) correspond to those in Tables 2 and S2. Actin (gene no. 7) mRNA was used as an internal standard

and SF revealed the presence of the same two SNPs found in A192-18 and CS-PMR1 (Fig. 5b). Based on these SNPs, we developed new molecular markers (CAPS-T86C and dCAPS-G99A) (Table S1; Fig. 6). We used 48 cucumber inbred lines to investigate the utility of these markers for MAS of the *zym* locus, and found that genotypes associated with both markers correlated with ZYMV resistance in all lines (Table 3).

Discussion

MAS for ZYMV resistance in cucumber

In cucumber, resistance to ZYMV is often inherited as a recessive allele at a single locus (*zym*; Kabelka et al. 1997). Using 128 $F_{2:3}$ families (A192-18 × CS-PMR1) and backcross populations (F_1 × A192-18 and F_1 × CS-PMR1), we also confirmed that ZYMV resistance in A192-18 was conferred by a single recessive locus (*zym^A192-18*). Therefore, MAS can improve breeding efficiency for ZYMV
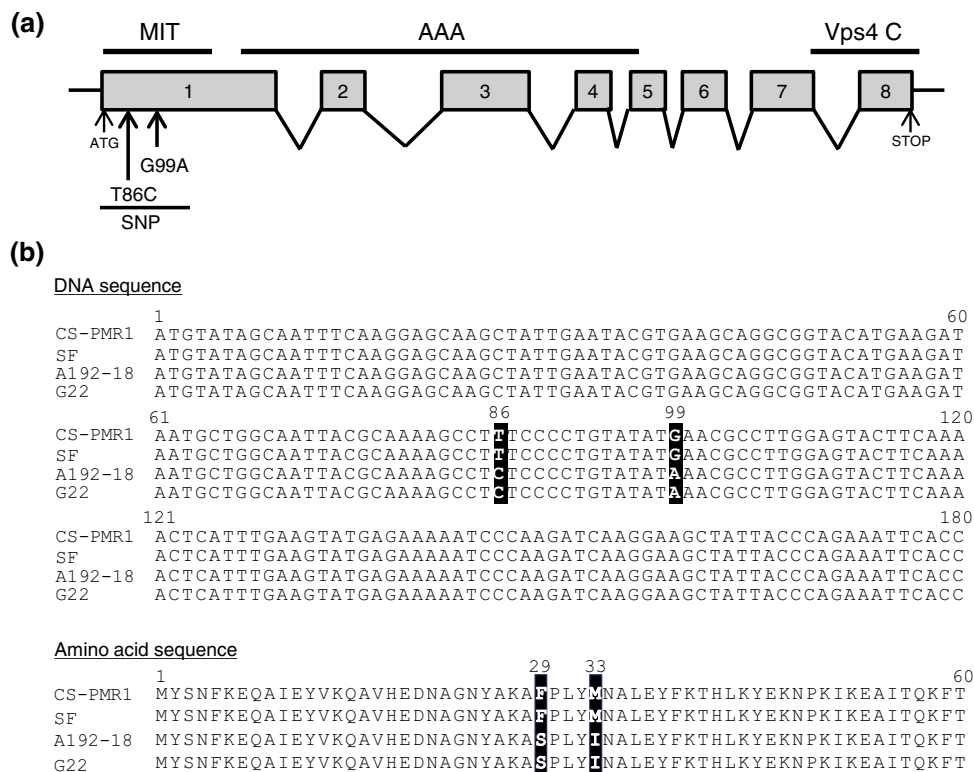

Fig. 5 VPS4-like gene and protein structures. **a** Exons are shown as *gray boxes* and introns as *lines*. Positions of MIT, AAA, and Vps4 C domains are displayed, and SNPs are indicated by *bold arrows*. **b** Alignments of nucleotide and amino acid sequences corresponding to a portion of the MIT domain in exon 1 from CS-PMR1, Sagami-

hanjiro fushinari (SF), A192-18, and G22 inbred lines. *Black shading* indicates the differences between ZYMV-resistant (A192-18 and G22) and susceptible (CS-PMR1 and SF) lines. DDBJ accession numbers are AB819727 (CS-PMR1), AB819728 (SF), AB819729 (A192-18), and AB819730 (G22)

Fig. 6 Restriction fragment length polymorphism revealed using **a** CAPS-T86C and **b** dCAPS-G99A markers in randomly selected F$_1$, F$_2$, and parental (C, CS-PMR1; A, A192-18) plants. Phenotypes were determined by inoculating seedlings with ZYMV Z5-1 (R, resistant; S, susceptible). Sizes of diagnostic bands are indicated on the *left*
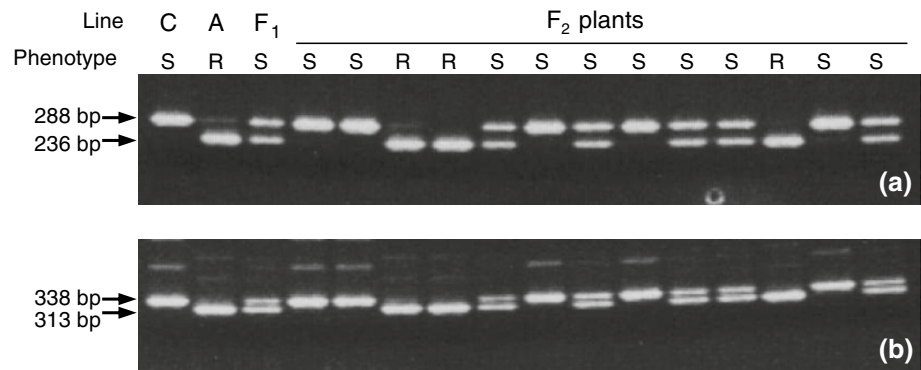

Table 3 Cucumber lines evaluated with the molecular markers CAPS-T86C and dCAPS-G99A for the $Zym^{A192-18}/zym^{A192-18}$ locus

	Line	Origin	Phenotype[a]	Genotype[b]			Line	Origin	Phenotype[a]	Genotype[b]	
			R/S	CAPS-T86C	dCAPS-G99A				*R/S*	CAPS-T86C	dCAPS-G99A
1	A122-1	Japan	*R*	A	A	25	HF221	Japan	*S*	B	B
2	A122-5	Japan	*R*	A	A	26	ST245-b	Japan	*S*	B	B
3	16AS	Japan	*R*	A	A	27	H129	Japan	*S*	B	B
4	S19	Japan	*R*	A	A	28	28KGT	Japan	*S*	B	B
5	T126	Japan	*R*	A	A	29	GH195	Japan	*S*	B	B
6	202 K	Japan	*R*	A	A	30	MV276	Japan	*S*	B	B
7	RJ2	Japan	*R*	A	A	31	ER23	Japan	*S*	B	B
8	KY23	Japan	*R*	A	A	32	86A	Japan	*S*	B	B
9	DZ2	Japan	*R*	A	A	33	97GG	China	*R*	A	A
10	JG12	Japan	*R*	A	A	34	234 K	China	*S*	B	B
11	AS53	Japan	*R*	A	A	35	56B	Korea	*S*	B	B
12	KS1	Japan	*R*	A	A	36	6GU	Iran	*R*	A	A
13	HE1	Japan	*R*	A	A	37	KY345	Iran	*R*	A	A
14	HO1	Japan	*S*	B	B	38	DSA21	India	*S*	B	B
15	S001	Japan	*S*	B	B	39	QW36	India	*S*	B	B
16	KA23	Japan	*S*	B	B	40	EDD231	USA	*S*	B	B
17	S56	Japan	*S*	B	B	41	TR1	USA	*S*	B	B
18	AU239	Japan	*S*	B	B	42	FR97	France	*S*	B	B
19	OK26	Japan	*S*	B	B	43	KMY90	The Netherlands	*R*	A	A
20	TA383	Japan	*S*	B	B	44	LKJU23	The Netherlands	*R*	A	A
21	SR12	Japan	*S*	B	B	45	GR291	The Netherlands	*R*	A	A
22	SG489	Japan	*S*	B	B	46	MY086	The Netherlands	*R*	A	A
23	AH214	Japan	*S*	B	B	47	WER2	The Netherlands	*S*	B	B
24	K395-a	Japan	*S*	B	B	48	DCT951	The Netherlands	*S*	B	B

[a] Inoculation test phenotype designation: *R*, resistant; *S*, susceptible

[b] Marker genotype designation: A, A192-18 allele ($zym^{A192-18}/zym^{A192-18}$); B, CS-PMR1 allele ($Zym^{A192-18}/Zym^{A192-18}$)

resistance in cucumber. We first constructed a framework genetic map using available cucumber molecular marker resources and published reference maps. The $zym^{A192-18}$ locus was located on Chr6, with its closest flanking markers separated by 2.2 cM (Chr6: 10781458–11069339). Using a large population of 2,429 F$_2$ plants, we identified 10 new polymorphic SSR markers flanking the $zym^{A192-18}$ locus in parental lines and constructed a high-resolution map of this locus. The physical distance between the closest flanking markers (UW080856 and UW080841) was <50 kb. One of the six candidate genes in this region, the VPS4-like gene (Chr6; 10850330–10855926), had two

SNPs in coding regions giving rise to amino acid substitutions. Validation in 48 cucumber inbred lines with codominant molecular markers CAPS-T86C and dCAPS-G99A—newly developed based on SNPs of the VPS4-like gene—showed consistency between genotypes and ZYMV resistance phenotypes. Park et al. (2004) also developed ZYMV resistance-linked DNA markers, but these were found to be monomorphic among the 48 cucumber inbred lines in our study (data not shown). Our developed molecular markers are thus additional effective tools for ZYMV resistance breeding in cucumber.

Recessive genes for ZYMV resistance in cucumber

Several recessive plant virus resistance genes have recently been determined to be mutated genes of eukaryotic translation initiation factors (Maule et al. 2007). Since we found no translation initiation factor genes within the 50-kb region flanking the $zym^{A192-18}$ locus, such translation factors can be excluded as candidates in cucumber. Drawing on the results of our high-resolution mapping, six candidate genes were identified from two databases. Semi-quantitative RT-PCR analysis revealed no differences in expression of these genes between A192-18 and CS-PMR1, suggesting that ZYMV resistance conferred by the $zym^{A192-18}$ gene is associated with amino acid mutations affecting its protein function rather than allelic transcription levels. Based on sequencing of candidate genes, the VPS4-like gene is the most likely candidate gene for the $zym^{A192-18}$ locus. In the *Tomato bushy stunt virus–Nicotiana benthamiana* experimental system, VPS4 has been reported to interact with endosomal sorting complexes (ESCRT) required for virus replication (Barajas et al. 2009). In our study, we found that both amino acid substitutions in the VPS4-like protein were in its MIT domain, which is known to interact with ESCRT-III subunits related to virus release and cell-to-cell transport in animal cells (Adell and Teisa 2011). Although the mechanism of interaction between ZYMV and the VPS4-like protein involved in conferring susceptibility is unclear, we suggest that ZYMV resistance due to the $zym^{A192-18}$ gene may be associated with replication and cell-to-cell movement of ZYMV via endosomal sorting.

With respect to the *zym* locus in cucumber, two different alleles (zym^{TMG-1} and zym^{Dina-1}) were present according to an allelism test (Kabelka et al. 1997), and a genetic map that includes the zym^{TMG-1} locus has been reported (Park et al. 2000). It is difficult to directly compare positions of $zym^{A192-18}$ and zym^{TMG-1} loci using our map and that of Park et al., however, as most molecular markers mapped around the zym^{TMG-1} locus were amplified fragment length polymorphism (AFLP) markers (Park et al. 2000). A YCZ-5 genomic clone, which covers DNA markers linked to the zym^{TMG-1} locus (Park et al. 2004), could potentially be used

for mapping onto our framework map. Our newly developed Indel marker (T1), located near zym^{TMG-1}-linked DNA markers (<12 kb), clustered with the $zym^{A192-18}$ locus in our framework map. The DNA region of genomic clone YCZ-5 was located on Chr6 (10928225–10933458) approximately 77 kb away from the $zym^{A192-18}$ candidate gene (VPS4-like gene; 10850330–10855926) based on the ICuGI database. Although this information is insufficient to elucidate the relationship among the three different *zym* genes, it suggests that $zym^{A192-18}$ is probably linked to zym^{TMG-1} and zym^{Dina-1}.

In conclusion, we developed new molecular markers and identified a candidate gene at the $zym^{A192-18}$ locus for ZYMV resistance in cucumber. To ascertain whether the three different *zym* genes are linked to each other and determine if the VPS4-like protein gene is equivalent to $zym^{A192-18}$, we will focus on genetic allelism test among the different *zym* genes and validation of the VPS4-like gene using a functional complementation approach involving either *Agrobacterium tumefaciens*-mediated transformation (Nanasato et al. 2012) or a virus-induced gene silencing system with *Apple latent spherical virus* vectors (Igarashi et al. 2009).

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