Expression Pattern Analysis of Four *Mlo* Genes from Rose

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Abstract. Rose (*Rosa hybrida*) is one of the most economically important ornamentals worldwide. Powdery mildew (*Podosphaera pannosa*) is a major disease in cut and potted roses. In dicots such as arabidopsis (*Arabidopsis thaliana*), pea (*Pisum sativum*), and tomato (*Solanum lycopersicum*), loss-of-function mutations in mildew resistance locus o (*Mlo*) genes confer high levels of broad-spectrum resistance to powdery mildew. Here, we present spatiotemporal expression patterns of four *Mlo* genes from *R. hybrida* based on real-time fluorescence quantitative polymerase chain reaction (qPCR). Phylogenetically closely related *R. hybrida* mildew resistance locus o (*RhMLO*) genes showed similar or overlapping tissue specificity and analogous responsiveness to external stimuli. *RhMLO1* and *RhMLO2* transcriptional levels were upregulated more than 2-fold by external stimuli, especially by inoculation with powdery mildew fungus *P. pannosa* at early time points. This phenomenon was not found for *RhMLO3* or *RhMLO4*. The results indicated that *RhMLO1* and *RhMLO2* might play important roles in rose–powdery mildew pathogen interactions. Our findings may provide useful information for the study of mechanisms of powdery mildew susceptibility in rose.

Powdery mildew is one of the most serious rose diseases. Nearly 40% of the fungicide sprayed on cut and potted roses is intended to control powdery mildew (Linde and Shishkoff, 2003). It can cause distortion and death of leaves and shoots, but even a mild case makes plants unsightly (Linde and Shishkoff, 2003). Disease control is economically important for cut and potted roses, which are almost exclusively produced in greenhouses throughout temperate regions or at higher elevations in the tropics. Powdery mildew in roses is caused by the obligate biotrophic ascomycete *Podosphaera pannosa* [formerly *Sphaerotheca pannosa* (Linde and Shishkoff, 2003)]. *P. pannosa* belongs to the family Erysiphaceae, which consists of other important powdery mildews such as *Blumeria graminis* of cereals (Gramineae). The pathogen species show considerable variation in pathogenicity (Leus et al., 2006; Linde and Debener, 2003).

Some strategies to develop powdery mildew-resistant crops have relied on the gene-for-gene resistance approach, which depends on the identification of a resistance gene in the host specific to a corresponding avirulence gene in the pathogen (Flor, 1971). Another strategy is based on a loss-of-function mutation in the mildew resistance locus o–based (*Mlo*) gene. *Mlo* resistance was characterized several decades ago in barley (*Hordeum vulgare*) as a recessively inherited trait that is effective against all known races of barley powdery mildew [*B. graminis* f.sp. *hordei* (Jorgensen, 1992)]. The MLO protein belongs to a plant-specific family of membrane proteins that contains seven transmembrane helices and a C-terminal calmodulin-binding domain (CaMBD) (Devoto et al., 1999; Kim et al., 2002). In dicots such as arabidopsis, pea, tomato, and peach (*Prunus persica*), loss-of-function mutations in *Mlo* genes confer high levels of broad-spectrum resistance (Bai et al., 2008; Consonni et al., 2006; Humphry et al., 2011; Jiwan et al., 2013; Pavan et al., 2011). Therefore, *Mlo* genes provide a general route by which to achieve highly effective race-independent resistance in angiosperms.

So far, four *Mlo* homologs *RhMLO1–RhMLO4* were isolated from rose leaves (Kaufmann et al., 2012). Phylogenetic analysis showed that all *Mlo* genes from dicots for which a function in powdery mildew–host interaction has been experimentally

demonstrated fall into one phylogenetic clade. The clade consists of two smaller clades, one consists of rose genes *RhMLO3* and *4*, and the arabidopsis genes *AtMLO2*, *6*, and *12*; the other consists of rose genes *RhMLO1* and *2*, and pea gene *PsMLO2* (Kaufmann et al., 2012). All four rose genes fall into the dicot cluster for powdery mildew–related *Mlo*s and are therefore candidate *Mlo* orthologs. Gene expression analysis has become increasingly important in many fields of biological research. Understanding patterns of expressed genes is expected to provide insight into complex regulatory networks and will most likely lead to the identification of genes relevant to new biological processes or that are implicated in disease (Vandesompele et al., 2002). Real-time quantitative PCR (RT-qPCR) has become the gold standard for the quantitative analysis of nucleic acids because of its high sensitivity, adequate reproducibility, broad quantification range, and ease of use. RT-qPCR is the most prominent gene expression quantification technique to date (Derveaux et al., 2010).

Here, the aim of our study was to obtain better insight into the characteristics of *Mlo* family members in rose by RT-qPCR. Phylogenetically closely related *RhMLO* genes showed similar or overlapping tissue specificity and analogous responsiveness to external stimuli. Our findings will help us to understand the role of the four *RhMLO* genes in response to biotic and abiotic stresses.

Materials and Methods

PLANT MATERIALS AND TREATMENT. The four *Mlo* genes [*RhMLO1*–*RhMLO4* (GenBank accession nos. JX847131–JX847134)] were isolated from *Rosa multiflora* hybrids, and *R. hybrida* 'Fentuan' (Tang, 2009) possesses these genes based on our preliminary work. Therefore, *R. hybrida* 'Fentuan' was employed as the plant material for gene expression pattern analysis. Three clones of 'Fentuan' (2 years after cutting propagation) were cultivated in a greenhouse under a 16-h light/8-h dark cycle, with a constant temperature of 22 °C. Roots were induced on stem cuttings in hydro-culture according to Mutui et al. (2005). Calli were induced according to Ding (2012). Tender leaves (folded leaves), adult leaves (the third to fifth unfolded leaves from the shoot apex), old leaves (the last unfolded leaves from the shoot apex), stems (nonlignification), buds (0.5 cm diameter), petals (from blooming flowers), flowers (blooming flowers), roots (taproots and lateral roots), and calli (1-month old) were collected from the three separate clones.

Several stress treatments were conducted on the third to fifth unfolded leaves from the shoot apex. The leaflets were placed in plastic boxes on water-saturated tissue paper. Here, some leaflets were collected before being treated or inoculated as controls. For wounding, the leaves were cut with a scalpel and stored for 2 h at 20 °C. Heat stress and cold stress were applied by placing the wet plastic boxes at 42 °C for 1 h and at 4 °C for 12 h, respectively. Two monospore isolates of *P. pannosa*, isolate MC and isolate YF, both from Kunming, China (Qiu et al., 2015), were maintained in vitro as described by Linde and Debener (2003). Pathogen stress was induced by the inoculation of leaflets with the two mixed compatible isolates at a density of 10^4 conidia/mL and incubated for 6, 12, 24, 36, 48, 72, and 96 h at 20 °C as described by Yan et al. (2006).

Three replicates were collected for each tissue and each treatment. All samples were immediately frozen in liquid nitrogen after harvest and stored at –80 °C for less than 1 month before RNA extraction.

TOTAL RNA EXTRACTION AND CDNA SYNTHESIS. Total RNA was extracted from 30 to 50 mg of each sample using TransZol Plant (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The remaining DNA was removed by DNase treatment using a DNA-free Kit (Invitrogen, Shanghai, China). The RNA concentration was assessed spectrophotometrically at 260 nm and was checked for purity by determining the optical density (OD) 260/280 nm and the OD 260/230 nm ratios. The RNA quality was assessed by gel electrophoresis and for a subset of samples with a Bioanalyzer 2100 using the RNA 6000 Nano Laboratory-Chip Kit (Agilent Technologies, Basel, Switzerland). For each sample, 500 ng of total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

SELECTION OF REFERENCE GENES AND PCR PRIMER DESIGN. Based on work by Klie and Debener (2011), two reference genes, protein phosphatase 2A (*PP2A*) and ubiquitin-conjugating protein (*UBC*), were selected for the expression analysis of different tissues, and SAND-family protein (*SAND*) and *UBC* were selected for the analysis of stressed leaves (Table 1). Four *RhMLO* genes were analyzed for secondary structures by the

Table 1. Primer sequences, product length, and reaction efficiency of *Rosa hybrida* mildew resistance locus o-1–4 (*RhMLO1*–*RhMLO4*) and three reference genes in real-time quantitative PCR.

Gene	GenBank accession no.	Primer sequence (5′-3′)	Amplicon length (nucleotides)	Efficiency (%)
RhMLO1 (*Rosa hybrida* mildew resistance locus o-1)	JX847131	F:TTAGGAAGAGCCAAGATGAGA R:CCGCAAGGTCAAGTAATCAAC	225	101.5
RhMLO2 (*R. hybrida* mildew resistance locus o-2)	JX847132	F:TCTTTGCGTGGACTTGGTATG R:GGAACTGAATGCTTGCTCTG	256	109.8
RhMLO3 (*R. hybrida* mildew resistance locus o-3)	JX847133	F:ATGGAACTTGAATATGAGCG R:CGATTGGATCTTGTAGAACTG	235	108.6
RhMLO4 (*R. hybrida* mildew resistance locus o-4)	JX847134	F:GACCCTCAATGTAAAAAACATGA R:TAGGAGCAAGGACAGGAATC	222	90.9
PP2A (protein phosphatase 2A)	JN399224	F:TGTCACTGCATCAAAGGACAG R:GACGAATTGTCTTCTCCACCA	110	101.8
SAND (SAND-family protein)	JN399228	F:GTGTTGAGGAGTTGCCTCTTG R:AACCTGTCGGGAGAATCTGTT	97	101.7
UBC (ubiquitin-conjugating protein)	JN399227	F:GCCAGAGATTGCCCATATGTA R:TCACAGAGTCCTAGCAGCACA	109	108.4

UNAFold (version 3.4; RNA Institute, Albany, NY) software (Bustin et al., 2009). Because each rose *Mlo* gene has more than one allele (Kaufmann et al., 2012), the primers for each *Mlo* gene were designed to match only one allele (their sequences are the same as GenBank no. JX847131–JX847134) using Beacon Designer (version 8.0; Premier Biosoft International, Palo Alto, CA) software with the following specifications: optimal annealing temperature at 58 ± 2 °C, optimal primer length of 18–24 nucleotides, optimal amplicon length of 80–300 bp, content of guanine and cytosine between 45% and 55%. OligoAnalyzer (version 1.0.2; Integrated DNA Technologies, Coralville, IA) was used to control for dimer and hairpin formation. The primers were highly specific for one allele of each gene as confirmed by a Blast online search (National Center for Biotechnology Information, 2014). The performance of the primers was tested in PCR reactions with both genomic DNA and complementary DNA (cDNA) as substrates. The sequencing results of PCR products showed a 100% match to the specific range of sequences in the GenBank accession for each rose *Mlo* gene.

RT-qPCR and data analyses. The amplification reactions were performed in transparent 0.1-mL, 96-well plates (Bio-Rad) using EvaGreen detection chemistry and run on the CFX96 system (Bio-Rad). The reactions were prepared in a total volume of 20 μL containing 2 μL of template, 1 μL of each primer [0.25 nM], 10 μL of SsoFast EvaGreen Supermix (Bio-Rad), and 6 μL of nuclease-free water. The water-only controls included 2 μL of nuclease-free water instead of a cDNA template and were run for each primer pair on each plate. The cycling conditions were set as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles of 10 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, + Plate Read. The efficiency of each primer pair was analyzed with five serial 5-fold dilutions of cDNA of leaves and calculated from the slope of the regression line of the quantification cycle (Cq) vs. the relative concentration of cDNA (Pfaffl, 2001). The amplification specificity for each primer pair was tested by a melting curve analysis ranging from 65 to 95 °C with temperature steps of 0.5 °C [CFX Manager™ Software Help Introduction (Bio-Rad)]. Two replicate amplifications were done for each cDNA.

The data were analyzed by CFX Manager Software (Bio-Rad). When a control sample (control) was assigned, the relative quantity (RQ) for any sample of a gene of interest (GOI) was calculated with the following formula according to Hellemans et al. (2007): Efficiency (E) = (% Efficiency × 0.01) + 1; $RQ_{sample(GOI)} = E_{GOI}^{(Cq_{control} - Cq_{sample})}$. $Cq_{control}$ = average Cq for the control sample; Cq_{sample} = average Cq for any samples with a GOI.

Normalized expression (NE) is the relative quantity of the target gene normalized to the quantities of more than one reference gene. Reference target (ref) in a run that includes one or more reference targets in each sample. The calculation for NE was described in the following formula according to Vandesompele et al. (2002) and Hellemans et al. (2007), and it uses the RQ calculation:

$$NE_{sample(GOI)} = \frac{RQ_{sample(GOI)}}{\left(RQ_{sample(ref1)} \times RQ_{sample(ref2)} \times \ldots \times RQ_{sample(refn)}\right)^{\frac{1}{n}}}.$$

Statistical analysis of variance (ANOVA) was performed using SAS (version 9.0; SAS Institute, Cary, NC) software.

Standard errors of the mean was calculated and represented as in Figs. 1 and 2 and Table 2 (Joseph et al., 2010).

Results

Primer specificity and efficiency of RT-qPCR. The amplification specificity for each primer pair was tested by a melting curve (Supplemental Fig. 1). Each primer pair yielded a single-peak melt curve without nonspecific products, which indicated that the specificity was very high and the credibility of the RT-qPCR products was also very high.

PCR amplification efficiency was established by a standard curve (Supplemental Fig. 2). The mean primer efficiency varied from 90.9% for *RhMLO4* to 109.8% for *RhMLO2* (Table 1), which was in the range of the effective amplification efficiency scope of 90% to 110% [CFX Manager Software Protocols (Bio-Rad)].

Expression patterns in different tissues. For the determination of *RhMLO* expression in different tissues, we used the reference genes *PP2A* and *UBC* as internal controls. Expression levels in different tissues were calculated relative to the expression levels in root samples. The expression levels of the four *RhMLO* genes in nine different tissues are shown in Fig. 1.

None of the four genes are highly expressed in roots, stems, or calli, and all of the genes have the highest expression in leaves (with expression in younger leaves being higher than in older leaves). *RhMLO1* to *RhMLO3* are also, to varying degrees, expressed at intermediate levels in other tissues, whereas *RhMLO4* is expressed at very high levels in leaves but at very low levels in all other tissues.

Expression pattern in stressed leaves. To analyze stressed leaves, we used the two reference genes *SAND* and *UBC* as internal controls. Expression levels in stressed leaves were calculated relative to the expression level in untreated leaves. The expression levels of the four *RhMLO* genes in leaves that underwent different abiotic and biotic stresses are shown in Table 2 and Fig. 2. Both *RhMLO1* and *RhMLO2* exhibited similar expression patterns in stressed leaves: their expression levels increased more than 2-fold on treatment with different abiotic stimuli. Furthermore, these transcripts were also upregulated more than 2-fold at time points of 6, 12, 24, 36, and 48 h postinoculation (HPI) with *P. pannosa*.

Compared with *RhMLO1* and *RhMLO2*, both *RhMLO3* and *RhMLO4* showed opposite expression patterns after biotic or abiotic stress. Their expression levels decreased more than 2-fold on treatment with abiotic stimuli, and they were downregulated upon inoculation with *P. pannosa*.

Discussion

Gene expression pattern research is crucial for the elucidation of gene function in modern molecular biology. The spatiotemporal expression pattern of a certain gene is closely related to its function, so identifying the expression pattern of a gene may be an important step in explaining its function and the underlying mechanism. *Mlo* genes have been isolated from several species based on their similarity to well-characterized genes from barley and arabidopsis. However, only a few of these sequences have been functionally characterized and shown to be involved in plant–powdery mildew interactions by further experiments (Cheng et al., 2012; Feechan et al., 2008; Jiwan et al., 2013; Liu and Zhu, 2008; Yu et al., 2005;
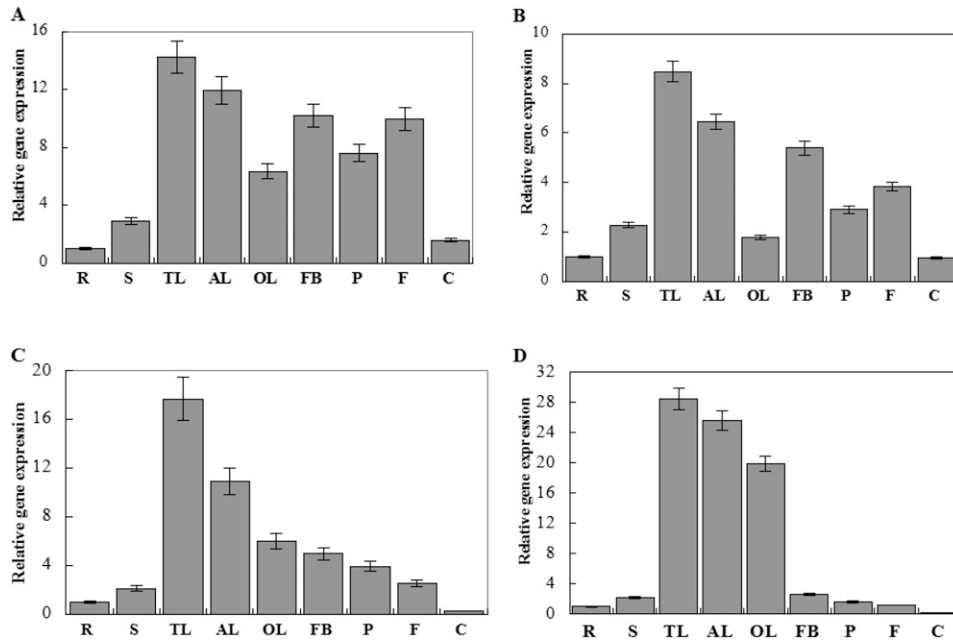
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Fig. 1. Relative expression levels of *Rosa hybrida* mildew resistance locus o-1–4 [*RhMLO1–RhMLO4* (**A–D**)] in nine tissues of *R. hybrida* 'Fentuan' based on real-time quantitative PCR. Two reference genes, protein phosphatase 2A (*PP2A*) and ubiquitin-conjugating protein (*UBC*), were internal control genes. The expression level in roots was used as a baseline (1.00) for calculating relative expression levels in other tissues. Data are shown as the means ±SE. R = roots; S = stems; TL = tender leaves; AL = adult leaves; OL = old leaves; FB = flower buds; P = petals; F = flowers; C = calli.
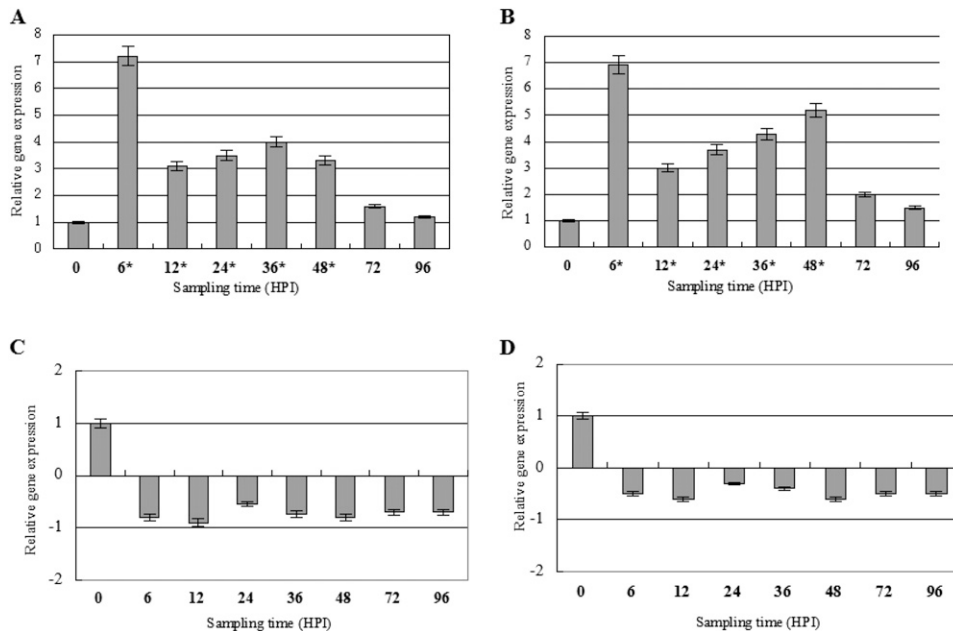

Fig. 2. Relative expression levels of *Rosa hybrida* mildew resistance locus o-1–4 [*RhMLO1–RhMLO4* (**A–D**)] in leaves of *R. hybrida* 'Fentuan' inoculated with *Podosphaera pannosa* (*Pp*) based on real-time quantitative PCR. Leaves were sampled at 6, 12, 24, 36, 48, 72, and 96 h post-inoculation (HPI). Two reference genes, SAND-family protein (*SAND*) and ubiquitin-conjugating protein (*UBC*), were used as internal control genes. The expression level of uninoculated leaves (0 HPI) was used as the baseline (1.00) for calculating relative expression levels in leaves inoculated with *Pp* at different stages. Data are shown as the means ±SE. Significant differences compared with untreated leaves (0 HPI) via analysis of variance (ANOVA) at $P \leq 0.05$ are indicated with an asterisk.

2008; Chen et al., 2006; Piffanelli et al., 2002; Zheng et al., 2013).

Phylogenetic analysis showed that RhMLO1 and RhMLO2 contain all of the sequence signatures considered to be diagnostic for MLOs. However, all sequence motifs were conserved, except that one mutation at the C-terminus in RhMLO3 and RhMLO4 altered the amino acids [S-F-S-F instead of D/E-F-S-T/F (Kaufmann et al., 2012)]. Therefore, to better understand the functional characteristics of these four genes, an analysis of their spatiotemporal expression patterns is necessary. Here, we presented the expression patterns of *RhMLO* genes both in different tissues and after several biotic and abiotic stress stimuli of *R. hybrida* 'Fentuan'.

The molecular phylogenetic tree containing *Mlo* genes of other species was constructed by Kaufmann et al. (2012). *RhMLO1* and *RhMLO2* clustered together with the *MLO1* gene from *P. sativum*, while *RhMLO3* and *RhMLO4* were in a separate cluster with arabidopsis *MLO2*, *MLO6*, and *MLO12*. The transcriptional levels of phylogenetically closely related *RhMLO* genes showed similar or overlapping tissue specificity and analogous responsiveness to external stimuli. *RhMLO1* and *RhMLO2* transcript levels were higher in young leaves and floral buds than in other tissues. The expression levels in those tissues may have a connection to susceptibility because of the susceptibility of those tissues (Tang, 2009). Piffanelli et al. (2002) found that the expression of *Mlo* genes from barley was upregulated by fungal infection and wounding. Chen et al. (2006) reported that the transcripts of *AtMLO2*, *AtMLO6*, and *AtMLO12* genes from arabidopsis were upregulated more than 2-fold by chemical, fungal, bacterial, and abiotic stresses. Bai et al. (2008) found that the transcript of *SlMLO1* from tomato was upregulated at time points of 6 and 36 HPI with tomato powdery mildew (*Oidium neolycopersici*). Shen et al. (2012) reported that the expression of *Mlo* from soybean

Zheng et al., 2013). These members are characterized by the presence of a tetrapeptide (D/E-F-S-T/F) motif in the cytoplasmic region at the C-terminus of the protein and their responses to powdery mildew infection at early time points (Bai et al.,

(*Glycine max*) was influenced by various phytohormone and abiotic treatments. Zheng et al. (2013) reported that the transcripts of *CaMLO2* from pepper (*Capsicum annuum*) were upregulated at time points of 5, 7, and 25 HPI with pepper powdery

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Table 2. Relative expression levels of *Rosa hybrida* mildew resistance locus o-1–4 (*RhMLO1*–*RhMLO4*) in different stressed leaves of *R. hybrida* 'Fentuan' based on real-time quantitative PCR.[z]

Treatments (in leaves)[y]	Relative gene expression (mean ± SE)[x]			
	RhMLO1	*RhMLO2*	*RhMLO3*	*RhMLO4*
Wounded (2 h)	**3.10 ± 0.14***	**3.03 ± 0.13***	−2.74 ± 0.14*	−5.90 ± 0.24*
Heated (1 h)	**3.07 ± 0.17***	**3.05 ± 0.11***	−3.33 ± 0.19*	−3.81 ± 0.20*
Cold (12 h)	**3.21 ± 0.12***	**3.32 ± 0.11***	−3.59 ± 0.21*	−4.11 ± 0.17*

[z]Two reference genes, SAND-family protein (*SAND*) and ubiquitin-conjugating protein (*UBC*), were internal control genes. The expression level in untreated leaves was used as a baseline (1.00) for calculating relative expression levels in different stressed leaves. Significant differences compared with untreated leaves are indicated with an asterisk ($P \leq 0.05$).

[y]For wounding, the leaves were induced with a scalpel and stored for 2 h at 20 °C. Heat stress and cold stress were applied at 42 °C for 1 h and at 4 °C for 12 h, respectively.

[x]Positive numbers in bold show they are upregulated in expression levels; negative numbers show they are down-regulated in expression levels.

mildew (*Leveillula taurica*). The abovementioned all candidate genes have been shown to be involved in resistance or susceptibility via complementation or gene knockout. As shown for candidate genes from other species, we found that transcript levels of *RhMLO1* and *RhMLO2* were significantly affected by biotic and abiotic stresses, especially by inoculation with *P. pannosa* at early time points (6–48 h). Taken together, our results indicate that *RhMLO1* and *RhMLO2* had similar expression patterns and were upregulated analogously by a variety of stimuli in our study.

Originally, *RhMLO3* and *RhMLO4* were thought to be functional candidate *Mlo* orthologs in rose based on phylogenetic analysis. Our results showed that *RhMLO3* and *RhMLO4* had a different expression pattern compared with the other two *MLO* genes. For example, their transcript levels were down-regulated by both biotic and abiotic stresses, and the levels decreased to less than 2-fold in leaves inoculated with *P. pannosa* compared with control leaves. These phenomena were also observed in some *Mlo* genes from other plants (Chen et al., 2006; Kim and Hwang, 2012; Shen et al., 2012), and some of these genes were shown to play other roles, i.e., as negative regulators of abscisic acid signaling (Lim and Lee, 2014). In addition to their corresponding proteins having a different sequence (S-F-S-F) in the cytoplasmic C-terminus (Kaufmann et al., 2012), the two candidate genes *RhMLO3* and *RhMLO4* were not obviously transcriptionally regulated by *P. pannosa* at early time points after infection, so they are not likely involved in susceptibility to powdery mildew. However, they were significantly transcriptionally down-regulated after abiotic stimuli, indicating that they may have other functions.

Our present work demonstrated that *RhMLO1* and *RhMLO2* may play important roles in the interactions between rose and *P. pannosa*. The discovery of these two genes may provide useful information for the study of mechanisms of powdery mildew susceptibility in rose. We plan to verify the functionality of these genes regarding the mediation of susceptibility to *P. pannosa* using transgenic technology. Recently, Wang et al. (2014) developed hexaploid wheat (*Triticum aestivum*) plants that exhibited heritable broad-spectrum resistance to wheat powdery mildew (*B. graminis* f.sp. *tritici*) by mutation of three *Mlo* alleles using transcription activator-like effector nuclease (TALEN) technology. We will attempt to generate roses with broad-spectrum resistance to *P. pannosa* based on TALEN or other technology in the future.

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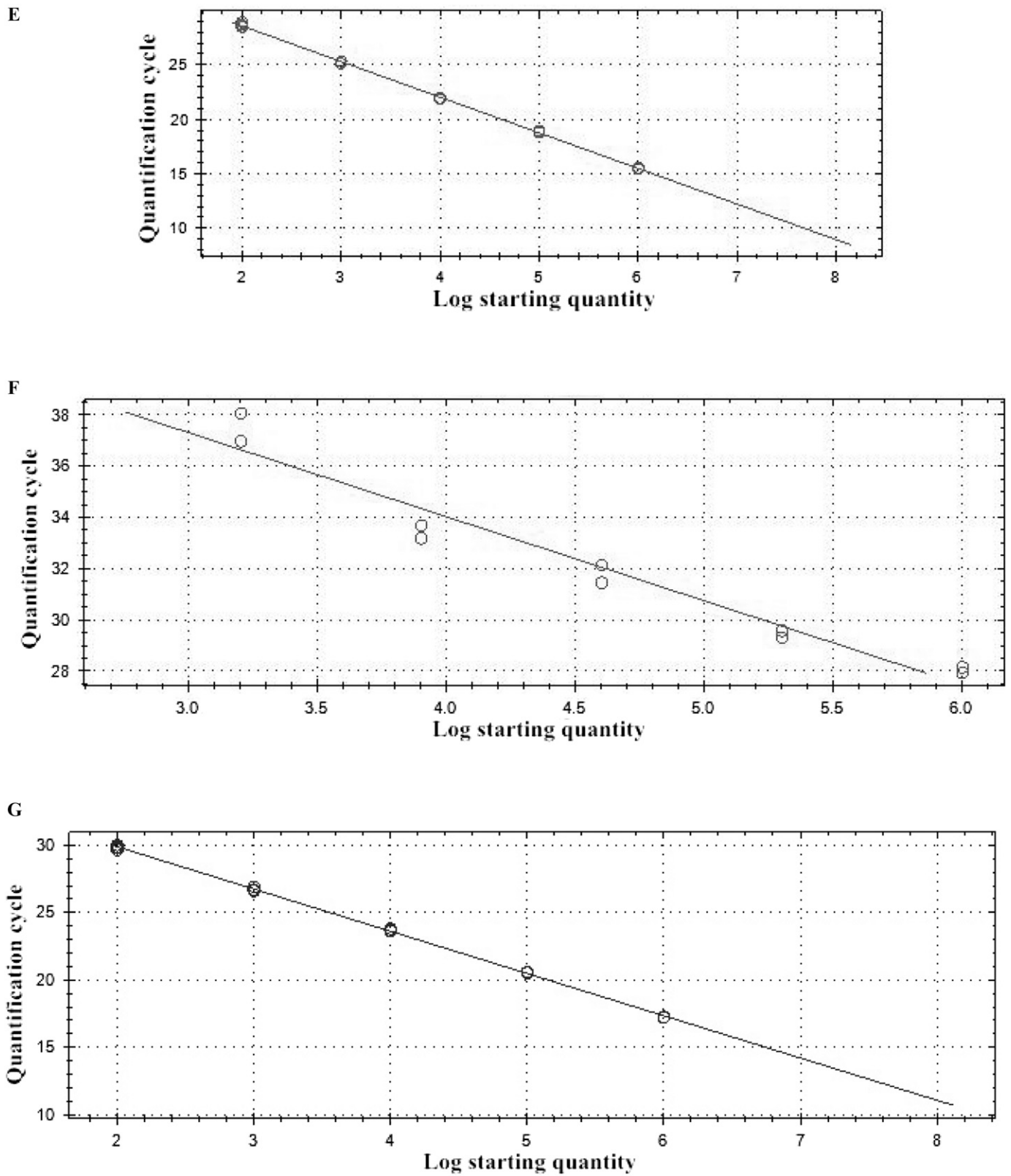
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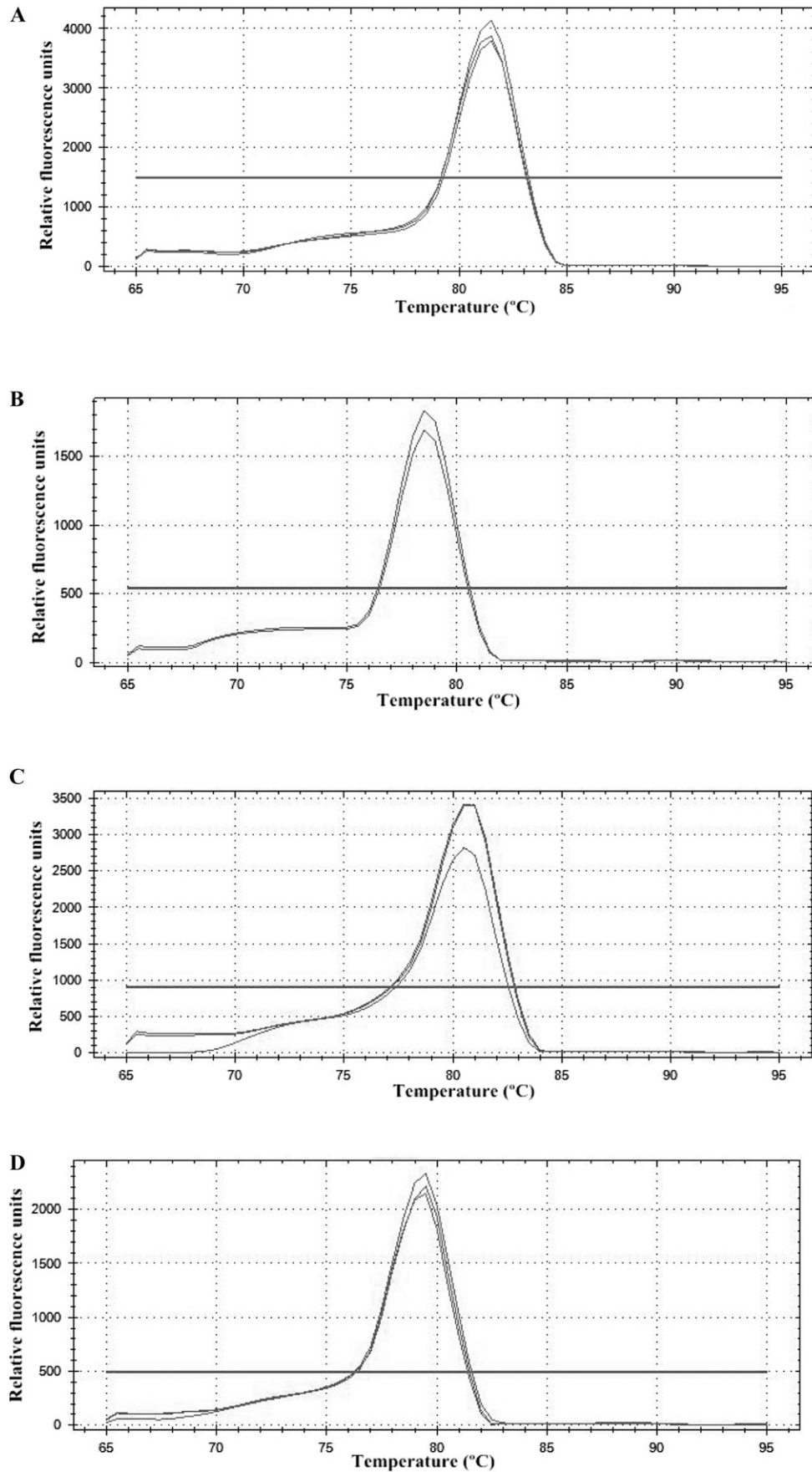
Supplemental Fig. 1. Amplicon specificity for each primer pair {*Rosa hybrida* mildew resistance locus o-1 to -4 [*RhMLO1–RhMLO4* (**A–D**)], protein phosphatase 2A [*PP2A* (**E**)], SAND-family protein [*SAND* (**F**)], and ubiquitin-conjugating protein [*UBC* (**G**)]} was tested by a melting curve analysis ranging from 65 to 95 °C with temperature steps of 0.5 °C (CFX Manager™; Bio-Rad, Hercules, CA).
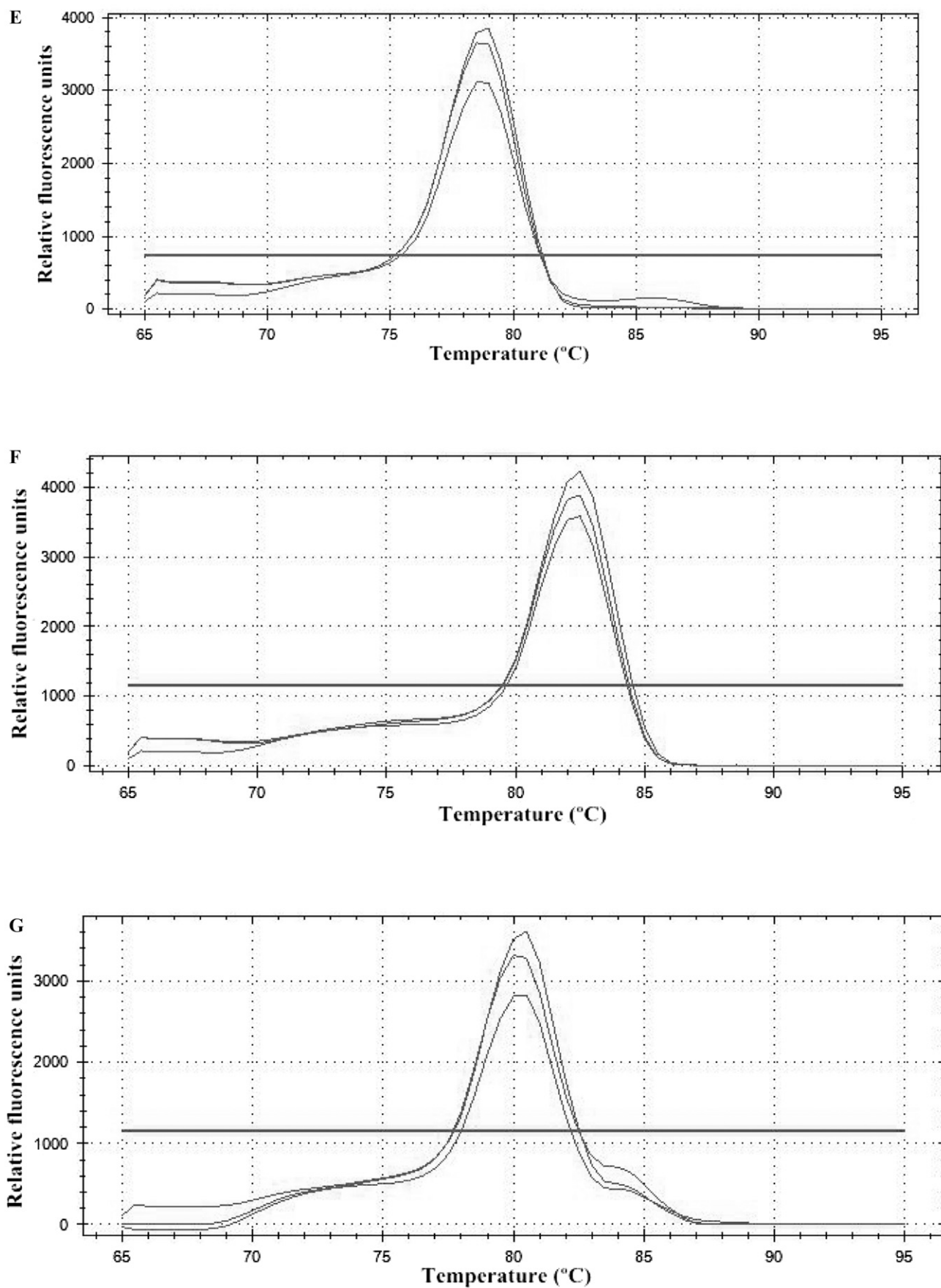
Supplemental Fig. 1. Continued.

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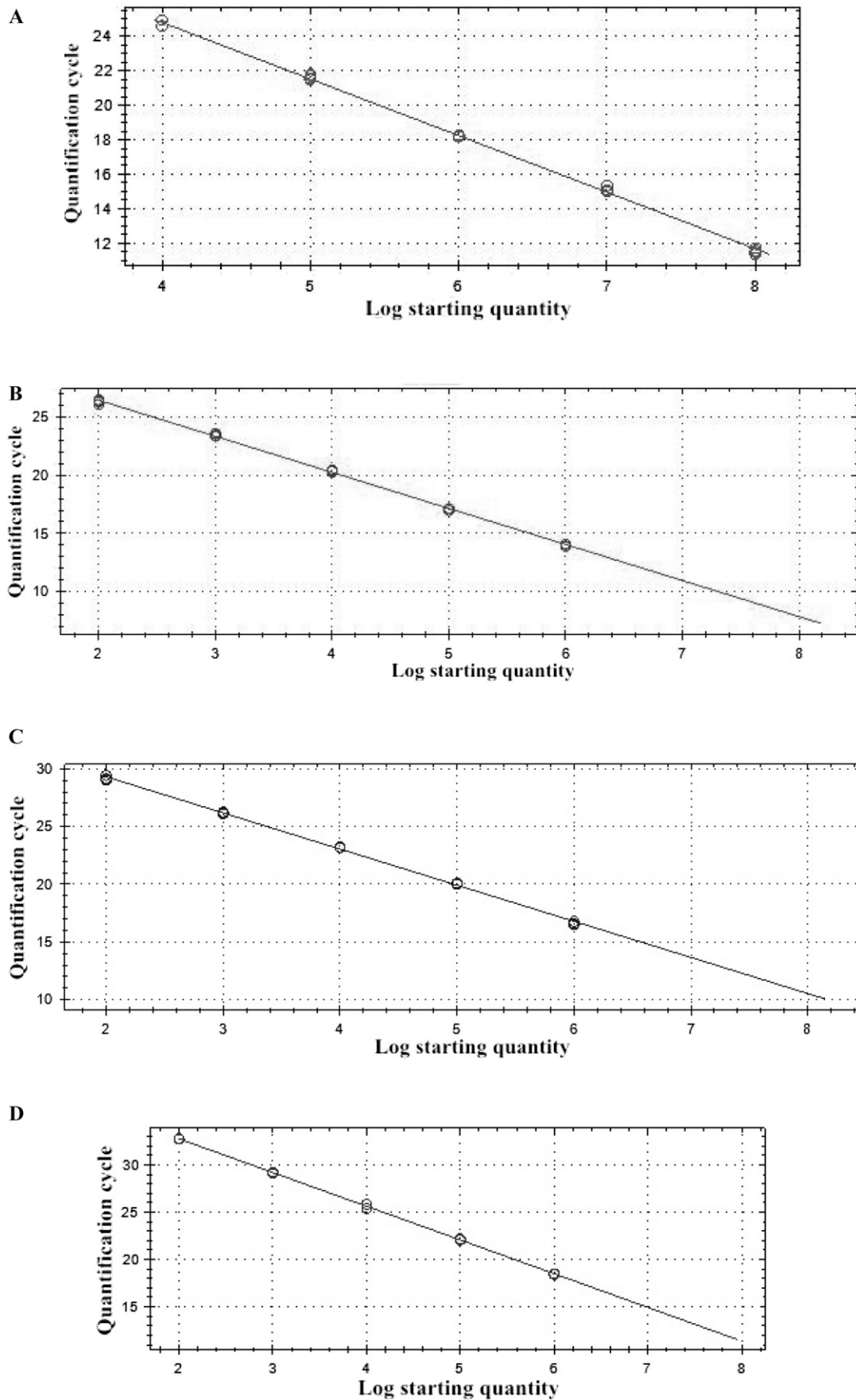
Supplemental Fig. 2. Amplicon efficiency of each primer pair {*Rosa hybrida* mildew resistance locus o-1 to -4 [*RhMLO1–RhMLO4* (**A–D**)], protein phosphatase 2A [*PP2A* (**E**)], SAND-family protein [*SAND* (**F**)], and ubiquitin-conjugating protein [*UBC* (**G**)]} was established using a standard curve (CFX Manager™; Bio-Rad, Hercules, CA).

E

F

G

Supplemental Fig. 2. Continued.