The \textit{I2} resistance gene homologues in Solanum have complex evolutionary patterns and are targeted by miRNAs

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Abstract

Background: Several resistance traits, including the \textit{I2} resistance against tomato fusarium wilt, were mapped to the long arm of chromosome 11 of Solanum. However, the structure and evolution of this locus remain poorly understood.

Results: Comparative analysis showed that the structure and evolutionary patterns of the \textit{I2} locus vary considerably between potato and tomato. The \textit{I2} homologues from different Solanaceae species usually do not have orthologous relationship, due to duplication, deletion and frequent sequence exchanges. At least 154 sequence exchanges were detected among 76 tomato \textit{I2} homologues, but sequence exchanges between \textit{I2} homologues in potato is less frequent. Previous study showed that \textit{I2} homologues in potato were targeted by miR482. However, our data showed that \textit{I2} homologues in tomato were targeted by miR6024 rather than miR482. Furthermore, miR6024 triggers phasiRNAs from \textit{I2} homologues in tomato. Sequence analysis showed that miR6024 was originated after the divergence of Solanaceae. We hypothesized that miR6024 and miR482 might have facilitated the expansion of the \textit{I2} family in Solanaceae species, since they can minimize their potential toxic effects by down-regulating their expression.

Conclusions: The \textit{I2} locus represents a most divergent resistance gene cluster in Solanum. Its high divergence was partly due to frequent sequence exchanges between homologues. We propose that the successful expansion of \textit{I2} homologues in Solanum was at least partially attributed to miRNA mediated regulation.

Keywords: \textit{I2} locus, Ty-2, Solanaceae, Evolution, Sequence exchange, miRNA

Background
Most of the disease resistance genes cloned from plant species encode nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains. The NBS-LRR encoding genes are often called disease resistance genes (or \textit{R} genes), since their main functions are protecting plants from pathogens with only a few exceptions, such as CHS3, a TIR-NBS-LRR-LIM encoding gene, playing role in cold stress and ADR1, a CC-NB-LRR encoding gene, involved in drought tolerance [1-4]. The \textit{R} genes belong to a large gene family, with dozens or hundreds of copies in a genome [5-7]. The \textit{R} proteins are composed of a variable N terminus, a conserved central NBS domain and a C-terminus with various number of short LRR motifs [8]. The N terminus usually has a Toll/interleukin-1 receptor (TIR) motif or a coiled-coil (CC) motif [9,10]. The NBS domains can bind and hydrolyze ATP or GTP, which are involved in signaling leading to pathogen resistance [11]. The LRR domains are most likely to be involved in recognition specificity [12,13].

\textit{R} genes tend to be clustered in plant genomes [6,9]. Many \textit{R} gene sub-families were shown to have been under diversifying selection, particularly at the hyper-variable solvent-exposed residues in the LRR region [14,15]. Consequently, \textit{R} genes represent the most divergent gene families in plant genomes [16]. Besides diversifying selection, frequent sequence exchanges among homologues have also contributed to the high divergence of \textit{R} gene families in plants [17,18]. However, \textit{R} genes vary dramatically in frequency of sequence exchanges. Some \textit{R} genes, termed Type I \textit{R} genes, had frequent sequence exchanges with

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homologues, resulting in extensive chimeric structures [15,18]. Frequent sequence exchanges may completely abolished allelic relationship of Type I R-genes from different genotypes. Different genotypes of the same species may harbor a various set of chimeric R-genes (Type I), leading to enormous distinct R-gene sequences in a population [19]. In contrast to the Type I R-genes, some R-genes (termed Type II) do not recombine with paralogues and are highly conserved in different genotypes of a species or closely related species. However, some Type II R-genes were found to be frequently lost in some genotypes, exhibiting presence/absence polymorphism [6,20]. The mechanism underlying the differentiation of above two distinct evolutionary patterns for R-genes remains unknown.

The resistance gene I2 in tomato (Solanum lycopersicum) encodes resistance against race 2 of the fusarium wilt pathogen Fusarium oxysporum f.sp lycopersici [21]. The I2 gene is a member of a gene cluster located on chromosome 11 of tomato. The I2 protein is a typical CC-type R protein. A resistance trait against tomato yellow leaf curl virus (TYLCV) was also mapped in the vicinity of the I2 locus though it remains unclear whether the trait is encoded by a I2 homologue [22,23]. I2 homologues are found in the syntenic region of potato (S. tuberosum); two resistance genes (R3a and R3b) against potato late blight have been cloned from this region of potato [24,25]; and at least nine additional R-genes against potato late blight were mapped to this locus, which harbors dozens of I2 homologues [24,26,27]. The R3a and R3b from potato exhibit 88% and 80% nucleotide identity with the I2 gene from tomato, respectively. I2 homologues were also found at the corresponding region in pepper (Capsicum annuum), and this locus may represent the most important R-gene locus in Solanaceae [28]. Previous studies of the I2 locus mainly focused on the cloning of functional R-genes, but the structure, evolution and gene content of the entire cluster remain unclear.

R-genes are believed to be physiologically toxic to plant cells and their expression is usually kept at low level when no pathogens exist [29,30]. Some R-genes are up-regulated when challenged by pathogens [31]. One mechanism to keep R-gene at low level in absence of pathogen is through miRNA mediated gene silencing [32,33]. Some R-genes could be cleaved by 22-nt miRNAs and successfully produce secondary small interfering RNAs (tasiRNAs) in a phased fashion (phasiRNA). The resistance gene R3a from potato was shown to be cleaved by 22-nt miR482 family and produce phasiRNA [33]. Whether the I2 family from tomato is regulated by miR482 or other miRNAs remains unknown.

In this study, we analyzed the structure and evolution of the I2 locus in tomato, and compared it with that in potato. First, I2 homologues were cloned from several genotypes of cultivated and wild tomato, including genotypes with resistance against TYLCV. Their evolution was investigated in detail through sequence analysis of I2 homologues from multiple genotypes of tomato, potato, pepper and tobacco. The miRNAs targeting I2 homologues in potato and tomato were investigated and compared. The role of miRNAs on R-gene expansion was discussed. The comprehensive study of the structure of the I2 locus may facilitate the cloning and the use of R-genes located in this region, and the evolutionary study may shed light on the mechanism of R-gene expansion in a genome.

**Methods**

**Tomato materials used in this study**

Since resistance gene Ty-2 against TYLCV was genetically mapped to the I2 locus, a commercial tomato hybrid cultivar Hongxiaoli with Ty-2 was included in this study. The hybrid was selfed and a F2 population with 736 individuals was generated. A homozygote Ty-2/Ty-2 (flanking markers are homozygous) was obtained from the F2 population to represent the Ty-2 (T) haplotype. Similarly, a susceptible homozygote of ty-2 was identified to represent the susceptible ty-2 (t) haplotype. The F2 population was used to map I2 homologues from the T and t haplotypes. The T haplotype was introgressed into cultivated tomato from S. habrochaites [23,34]. Four additional S. habrochaites accessions, LA1777, LA1740, LA2158 and LA2860, were also included in this study for I2 homologue analysis.

**Inoculation of TYLCV and phenotyping**

To phenotype above tomato genotypes on reaction to TYLCV, seedlings of 4–6 leaf stages were inoculated with a TYLCV agrobacterium infectious clone [35]. Inoculated seedlings were kept in a growth chamber under 16 h light/8 h dark cycle for a month before investigating symptom of TYLCV (curve leaf).

**PCR amplification, cloning and sequencing of I2 homologues**

The tomato genomic DNA were extracted from mature leaves of each genotypes using CTAB method, and genomic DNA were used as template to PCR amplify I2 homologues [36]. Nearly full-length (~3.5 kb) fragments of I2 homologues were amplified from aforementioned genotypes of tomato using a pair of degenerated primers (Table 1), which were located at +165 and +3,685 of the resistant gene I2, respectively. PCR amplification was in a 25 μl reaction with 1 unit Fast Pfu Taq (TransGen, Beijing, China), treated for 5 mins at 95°C, followed by 32 cycles at 95°C for 30 s, 52°C for 30 s, and a final extension at 72°C for 1.5 min. PCR products were gel
purified using Gel Purification Kit (Generay, Shanghai, China) and ligated into vector pZERO5 using TA cloning kit (TransGen, Beijing, China). Individual colonies were sequenced until no new genes were obtained in the last ten colonies. If sequences from different clones have higher than 99.7% nucleotide identity, they were considered to be derived from the same gene. The \( I_2 \) homologues amplified from an accession were named as accession name followed by \( I_2 \) then by a number, such as LA1777-I2-1. \( I_2 \) homologues from the T and t haplotypes were named as T-I2- and t-I2- followed by a number, respectively.

**Sequence analysis**

Four \( I_2 \) homologues [\( I_2 \) [GenBank: AF118127], \( I_{2C-1} \) [AF004878], \( I_{2C-2} \) [AF004879], \( I_{2C-5} \) [AF408704]], originated from wild tomato species \( S. pimpinellifolium \) were downloaded from GenBank [21,37,38]. Two partial genes \( I_{2C-3} \) and \( I_{2C-4} \) were excluded from this study. The sequences of \( R_{3a} \) [AY849382], \( R_{3b} \) [JF900492.1] and sixteen \( I_2 \) homologues [AY849383-AY849385, EF638450-EF638453, EF638455, EF638456, EF638458, EF638460-EF638465] from potato genotype SH83-92-488 were retrieved from GenBank [21,37,38]. Two partial genes \( I_{2C-3} \) and \( I_{2C-4} \) were excluded from this study. The sequences of \( R_{3a} \) [AY849382], \( R_{3b} \) [JF900492.1] and sixteen

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**Table 1 Primer sequences used for this study**

| Marker | Sequences 5’ to 3’ | Note |
|--------|-------------------|------|
| M-7300 | F:ATTCCACCCCTTGATGATGTT | Screening recombinant individuals |
|        | R:TTCCCTAGGCTAATCTCTCTTG | Restriction enzyme: Taq I |
| M-137  | F:TAGCTCCGTGCGATCCAT | Screening recombinant individuals |
|        | R:GTTCGTCTGCAATTTGCTATTA | Restriction enzyme: Mbo I |
| I2     | F:TTGCTGTAAGTGAYGCGASAGA | Amplification of \( I_2 \) homologs |
|        | R:TAGAGAGGRAGRGCAAT | |
| T-I2-1 | F:GCTTGATTTAGAGATATTGGAC | Mapping \( I_2 \) homologs |
|        | R:GGTGGAAATCTTGGCAGTCTCG | |
| T-I2-5 | F:CCCTCTCAAATGGGACCAACA | Mapping \( I_2 \) homologs |
|        | R:GGTGGAAATCTTGGCAGTCTCG | |
| T-I2-8 | F:ACACTAGCTGTTACCTTCTAT | Mapping \( I_2 \) homologs |
|        | R:TTCTCTTCATCTTTGTGTTC | |
| T-I2-2 | F:TTCCCTGCTACCTTTGTTGTTTCTC | Mapping \( I_2 \) homologs |
|        | R:CCAAAGTAACGTTCTGGCAATTT | Mapping \( I_2 \) homologs |
| T-I2-3 | F:GGCTTTAATTGGGCTCTGAGTCGATATG | Mapping \( I_2 \) homologs |
|        | R:AAATCCACCTCTTCACATGTAATTC | Mapping \( I_2 \) homologs |
| T-I2-6 | F:AGATGGAAGACATTAAATATCA | Mapping \( I_2 \) homologs |
|        | R:ATGTCATAGCTTGGCAGTCTTC | Mapping \( I_2 \) homologs |
| T-I2-7 | F:GTGCCTGTATGAGCTTCCAAGA | Mapping \( I_2 \) homologs |
|        | R:CGCATTTCTTCAACATGTAATTC | Mapping \( I_2 \) homologs |
| T-I2-9 | F:CCAATCCAGCTACCTTTCA | Mapping \( I_2 \) homologs |
|        | R:CAAGTTTTCCTGGAATTTTT | Mapping \( I_2 \) homologs |
| T-I2-10 | F:GTCCCCAAATCTTCCAGAG | Mapping \( I_2 \) homologs |
|        | R:CAAGTTTTCCTGGAATTTTT | Mapping \( I_2 \) homologs |
| Race-P1 | R: GGAGATCATTTGATGCTCAACATYA | Identification of miRNAs’ cleavage sites |
| Race-P2 | R: CGTGTCACACACATGCTTCC | Identification of miRNAs’ cleavage sites |
| Race-P3 | R: TTCTCCTCAATGGTACCTG | Identification of miRNAs’ cleavage sites |

The usage of primers is listed in ‘Note’ column. Restriction enzyme indicates the marker is a CAPS marker.

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Mapping of the I2 homologues in the T and t haplotypes
A F2 population with 736 individuals was used to map the I2 homologues from the T and t haplotypes. First, primers specific to each I2 homologue were designed (Table 1). A primer pair was considered as specific only when the target gene but none of the other genes in the T and t haplotypes was amplified by this primer combination. The gene-specific primers were used as markers to screen the F2 population and their linkage with CAPS markers M-73000 and M-137 at the I2 locus were analyzed.

Identification of miRNAs targeting I2 homologues
To detect miRNAs that potentially target I2 homologues, sequences of all I2 homologues were first used as query to screen for matching small RNAs (sRNA) from database SoMART (http://somart.ist.berkeley.edu/) [45]. Identified sRNAs with more than 100 reads in the database were further confirmed in the psRNATarget web server (http://plantgrn.noble.org/psRNATarget) using following parameters: maximum expectation = 5.0, and target accessibility - allowed maximum energy to unpair the target site (UPE) = 50. All confirmed matching sRNAs were mapped to tomato genome using bowtie [46]. Then, approximately 800 bp flanking sequences of all confirmed matching sRNAs were extracted, and their fold-back structures were predicted using the RNAfold program with default settings [46,47]. A folding structure was assumed if there was a central loop and a stem (matching structures were predicted using the RNAfold program). Matching sRNAs were extracted, and their fold-back structures were further investigated for approximately 800 bp flanking sequences of all confirmed matching sRNAs.

Results
The I2 locus spans more than 5 Mb on chromosome 11 of tomato and potato
Using BLASTN method, 36 I2 homologues were discovered in the sequenced genome of tomato cultivar Heinz 1706. Nineteen of them are located at the I2 locus, in a 5.4 Mb region on chromosome 11. Of the remaining 17 homologues, five are from other chromosomes and 12 are on chromosome 11 but away from the I2 locus, and they were excluded from further analysis (Additional file 1: Table S1). The positions of I2 homologues and important genetic markers around the I2 locus of tomato Heinz 1706 are shown in Figure 1. The sequenced genome of potato DM has 71 I2 homologues. Of them, 53 are located in the syntenic region of the I2 locus of tomato, spanning a region of at least 7.4 Mb (Additional file 1: Table S1). Fifteen I2 homologues are not on chromosome 11, and three I2 homologues are on chromosome 11 but far away from the syntenic region of the I2 locus. Therefore, the I2 locus was most likely expanded before the speciation of Solanum and may have also experienced amplifications after speciation.
**Comparative analysis of the I2/R3 locus in tomato and potato**

To investigate structure variation between tomato and potato, the I2 locus of tomato Heinz 1706 was compared with that of the potato genotype DM. Good co-linearity was found between tomato and potato, except in the centromeric part of the locus. However, the copy number of I2 homologues as well as their positions varies considerably between the two genomes (Figure 1).

Based on the distribution patterns of I2 homologues, this locus can be further divided into 9 sub-loci (sub-clusters). Dot plot analysis indicated that the duplications resulting in the 9 sub-loci were limited to the I2 sequences but not their flanking regions, since sequences flanking these I2 homologues are unrelated (Additional file 2: Figures S1 and S2). I2 homologues are present at six sub-loci of both tomato and potato genomes, though their copy number may vary dramatically (Figure 1). At the other three sub-loci (sub-loci 3, 4 and 9), I2 homologues are present in one but absent in the other genome, showing presence/absence divergence between the two species.

To investigate the genetic mechanism for such presence/absence divergence at the three sub-loci, their sequences were used to search the pepper genome. Sequences from the syntenic regions of sub-loci 3 and 4 but not 9 were found in pepper genome. Like in potato, the sub-locus 3 in pepper does not have any I2 homologues (Figure 2A), suggesting that the I2 homologue at sub-locus 3 of tomato was most likely gained after speciation. On the other hand, potato might have lost I2 homologues at sub-locus 4 after speciation since pepper has I2 homologues at this sub-locus (Figure 2B).

To study the evolution of I2 homologues from different sub-loci, a distance tree was constructed using approximately 2.5 kb sequences of I2 homologues (their 3’ parts were excluded due to various duplications) from
homologues from six tomato and potato genotypes, including the T and t haplotypes (see MM section), and four wild genotypes of *S. habrochaites* (LA1777, LA1740, LA2158 and LA2860). Inoculation with the infectious TYLCV clone showed that LA1777 is resistant to TYLCV, consistent with previous conclusion [52], while the other three wild genotypes are susceptible. To clone the *I2* homologues from these genotypes, a pair of degenerated primers (*I2-F/R*) was designed from the conserved regions of *I2* homologues (Table 1). Their PCR products were cloned and individual colonies were sequenced. A total of 75, 83, 48, 55, 70 and 70 colonies were sequenced, resulting in 8, 11, 13, 6, 13 and 11 distinct sequences from the T and t haplotypes, *S. habrochaites* accessions LA1777, LA1740, LA2158 and LA2860, respectively.

Mapping of the *I2* homologues in the T and t haplotypes
The 8 and 11 *I2* homologues obtained from the T and t haplotypes were genetically mapped using a F2 segregating population (736 individuals) derived from the hybrid cultivar Hongxiaoli. First, markers M-73000 and M-137, which flank the *I2* locus, were used to screen for recombinants. A total of 44 recombinants were obtained from the 736 F2 individuals. Specific primers were successfully designed for six of the 19 *I2* homologues from the T and t haplotypes, and all of them were fine mapped to sub-loci 5–7 of the *I2* locus (Figure 3).

Different evolutionary patterns for *I2* homologues
The 62 nearly full-length *I2* homologues obtained above were combined with the 10 *I2* homologues (>2.5 Kb) from Heinz 1706 and four homologues from wild species *S. pimpinellifilium* for further analysis [Genbank: KJ652840 – KJ652901] (Additional file 1: Table S1). A distance tree of the 76 *I2* homologues showed five distinguishable clades (Figure 3). Members from clade II and IV are highly conserved, with all pair-wise nucleotide identities >96.9%. Each genotype/haplotype usually has one representative in clades II and IV, and members within each clade are most likely alleles/orthologues. The exceptions are two *I2* homologues from LA1777 in Clade IV, and two *I2* homologues from LA1777 and LA1740 in clade II, respectively. The presence of two highly similar sequences is most likely due to the heterozygosity of the *I2* locus in these genotypes, which are self-incompatible. Only a few sequence exchanges were detected in clades II and IV, and mainly occurred between alleles (Additional file 1: Table S2). Therefore, clades II and IV have evolved independently from other homologues at the *I2* locus, with an evolutionary pattern similar to that of Type II *R*-genes [15].

In contrast, the other three clades in Figure 3 usually have multiple copies from one genotype and the average pair-wise nucleotide identity within a clade varies from 91.3% to 96.6%. Homologues within a clade are often equally related with each other and do not show obvious allelic/orthologous relationship. High sequence diversity...
and lack of allelic/orthologous relationship might have been attributed to frequent sequence exchanges among homologues [15]. To test above hypothesis, sequence exchanges were analyzed for all \(I2\) homologues in tomato using software Geneconv, and a total of 154 sequence exchanges were detected. The length of the sequence exchange tracts varied from 77 to 3,022 bp, with an average of 533 bp. Of them, 143 sequence exchanges occurred among members within a clade (98 within clade I, 7 within clade II, 8 within clade III, 1 within clade IV and 29 within clade V) (Additional file 1: Table S2). Clade I has homologues from three sub-loci (5, 6 and 7), and sequence exchanges happened between homologues from different loci. The frequent sequence exchanges and extensive chimeric structures suggest that the \(I2\) homologues in clades I and V have an evolutionary pattern of Type I \(R\)-genes [15].

Sequence exchanges occasionally occurred between Type I and Type II \(I2\) homologues or between different lineages of Type I genes

Eleven sequence exchanges were detected between genes from different clades. A sequence exchange of 162 bp was detected between \(LA1740-I2-5\) from clade I and three members (\(LA1740-I2-3\), \(LA2158-I2-5\), \(LA2860-I2-5\)) from clade II (Additional file 1: Table S2 and Additional file 2: Figure S4). The three members from clade II have identical sequences in the exchange tract. It is unlikely that the three genes had independent sequence exchanges in the same region. It is most likely that a 162 bp sequence of \(LA1740-I2-3\) converted gene \(LA1740-I2-5\), since these two genes are from the same genotype (LA1740). Similarly, gene \(LA1777-I2-1\) from clade II converted gene \(t-I2-2\) from clade I. Sequence exchanges might also occur between different lineages of Type I genes, such as between genes \(HZ-I2-10\) from clade I and \(t-I2-5\) from clade III. Interestingly, the directions of above sequence exchanges were unilaterally from Type II genes to Type I genes or between different lineages of Type I genes, but never from Type I genes to Type II genes.

Rare sequence exchanges among \(I2\) homologues in potato

Similar analyses (including phylogenetic analysis and sequence exchange) were applied to \(I2\) homologues in
potato. A total of 63 I2 homologues (>2.5 Kb, and one gene with many unknown nucleotides excluded) were obtained from Genbank, mainly from the sequenced genome of DM and the R3a haplotype of the diploid S. tuberosum (Additional file 1: Table S1). Three of them are not from the I2 locus and one has too many missing data, and they were excluded from further sequence analysis. A distance tree was constructed for the remaining 59 potato I2 homologues, and seven tomato homologues including I2, I2C-1 and one from each clade in Figure 3 were included for comparison. Consistent with their locations shown in Figure 1, all I2 homologues from tomato are grouped together with homologues from sub-loci 1–7 of potato (clade I in Figure 4). The homologues from clade I in Figure 3 are quite divergent (mostly < 90% nucleotide identity, with an average of 87.5%) but evenly related. Seven sequence exchanges were detected among the 34 I2 homologues from potato in clade I (Additional file 1: Table S2). Such pattern is in striking contrast to that for the I2 homologues from tomato, in which I2 homologues were differentiated into several groups, including both Type I and Type II genes (see above). No obvious differentiation (i.e. no well supported clades in Figure 4) among potato I2 homologues and occasional sequence exchanges suggest that the I2 homologues in sub-loci 1–7 in potato represent an ancient lineage of Type I genes that were originated at least 7 MYA before the divergence of tomato and potato [53,54].

The homologues from sub-loci 8 and 9 of potato form four clades (II, III, IV and V) in Figure 4. Ten and two sequence exchanges were detected among members in clade II and clade V, respectively. Members within these two clades exhibited 91.8% and 95.3% average nucleotide identity, respectively. Sequence exchanges among members of each clade and high diversity of I2 homologues suggest that clades II and V represent two distinct lineages of Type I I2 homologues [15]. The evolutionary pattern of clade III and IV remains unclear because only two sequences were obtained from this study.

Identification of miRNAs for the I2 homologues
The resistance gene R3a from potato was shown to be targeted by members of 22-nt miR482 family [33]. Computational analysis showed that I2 homologues from tomato may also be targeted by miR482 family, which has about 380 reads per million from three tissues (leaf, flower and fruit) of tomato (miRbase release 20). To investigate if I2 homologues from tomato are targeted by other miRNAs, the tomato sRNA libraries (http://somart.ist.berkeley.edu) were first searched using 76 I2 homologues. Consequently, a total of 1,439 distinct sRNAs, including the 22-nt sly-miR6024, were found in the sRNA database when five mismatches were allowed. Sly-miR6024 was previously shown to regulate the expression of R-gene Tm-2 in tomato [33]. To investigate if sly-miR6024 also target the I2 homologues, modified RNA ligase-mediated 5′-RACE was performed [50,51]. Sequencing the PCR products showed that the miRNAs of at least one I2 homologue from cultivar Hongxiaoli were cleaved at the predicted targeting site of miR6024 (Figure 5 and Additional file 2: Figure S5). However, no cleavage product was detected at the predicted target site of miR482. Therefore, the I2 homologues from tomato are targeted by miR6024 and may not be targeted by miR482. The cleavage of miR6024 on I2 homologues was also confirmed in a degradome database of tomato. Fourteen partial I2 cDNAs were identified in the database. One of partial ones starts from the 485th nucleotide of gene T-I2-3, confirming the cleavage function of miR6024 (Additional file 1: Figure S6A).

The targeting site of sly-miR6024 encodes the 206th – 213th amino acids in the I2 protein, partially overlapping with the conserved P loop. This sequence is highly conserved in all I2 homologues in tomato and potato. We hypothesize that miR6024 regulate expression of most, if not all I2 homologues in tomato, which may facilitate its expansion in a genome.

MiR6024 triggers 21-nt phased siRNA from I2 homologues
It was shown that 22-nt miRNAs often trigger the biogenesis of secondary phased siRNA [55,56]. To investigate if miR6024 can trigger phased siRNA, small RNAs from tomato were analyzed using program SoMART [45]. A total of 847 sRNAs were successfully mapped to a representative I2 homologue (T-I2-3). They were predominantly 21-nt in length and with 5′ U residue, consistent with the features of tasiRNA [33,57]. Most of them were mapped to the downstream of the cleavage site of miR6042 with a phased pattern (Additional file 2: Figure S6B). The structure of these sRNAs and their phasing with the miR6024 cleavage site indicated that they were tasiRNAs triggered by the 22-nt miRNA6024.

RACE-PCR and degradome database were used to investigate the potential regulating effects of tasiRNAs. Three potential cleavage sites of phasiRNA were identified. A degraded mRNA of gene T-I2-6 starting at 579th nucleotide and a degraded mRNA of gene T-I2-3 starting at 546th nucleotide, were obtained using RACE-PCR strategy (Additional file 2: Figure S6C). These two degraded mRNA were most likely the cleavage products of tasiRNAs (3′S5 and 3′S3, respectively) since their cleavage points are located in the middle of a tasiRNA triggered by the miRNA6024 (Figure 5). In addition, a degradome RNA from tomato degradome database was most likely generated by phasiRNA 3′S15 (Figure 5 and Additional file 2: Figure S6D).

MiR6024 was originated after the divergence of Solanaceae
To gain insight into the evolution of miR6024 in plants, four members of the miR6024 family were downloaded
Figure 4 Distance tree of I2 homologues from potato. Seven representative I2 homologues from tomato are included, which are all grouped into clade I. Genes with name “DM-I2-” are from potato cultivar DM; genes with name “SH-I2-” are from S. tuberosum; genes with name “HZ-I2-” are from tomato Heinz 1706. Numbers on nodes are bootstrap values, and values <65 are not shown.
from miRBase (release 20) including sly-miR6024 from tomato, nta-miR6024 from tobacco, stu-miR6024-3p and stu-miR6024-5p from potato. In addition, whole genome sequences of ten plant species were chosen to identify miR6024 genes using bioinformatic approach (see MM section). The miR6024 sequences were used to BLAST search the ten genomes, and significant hits were found in eight of the ten plant species except in C. papaya and A. lyrata. However, the flanking sequences of the significant hits in M. guttatus and V. vinifera could not form hairpin structure, and therefore these two species do not have miR6024. All the six Solanaceae species have the miR6024 sequence and its flanking sequences (miR6024 gene) can form a predicted hairpin structure. The miR6024 gene has no similarity with I2 homologues except that the miR6024/miR6024* can match the target site in I2 homologues. Therefore, the miR6024 was not originated from duplication and inversion of I2 sequences [33]. The 22-nt mature miR6024 was confirmed in a pepper sRNA database (Dr. Li, F., unpublished data). However, point mutations were observed in the miR6024 region of N. sylvestris and N. benthamiana, and it remains unclear if these mutations affect the biogenesis of miR6024 in the two species (Figure 6). Since miR6024 is present in distantly related species in Solanaceae, we hypothesize that the miR6024 was originated in the common ancestor of the Solanaceae family.

### Discussion

#### Divergence of I2 locus among different Solanaceae species

The I2 locus is a hotspot for R-genes, with several qualitative resistance traits (such as I2, Ty-2 and Sm from tomato, R3, R6 and R7 from potato, and L from pepper) and quantitative resistance traits mapped to this locus. A good understanding of the structure and evolution of this locus will facilitate the cloning and efficient use of R-genes from this region.

The I2 locus contains multiple homologues and spans several megabases on the long arm of chromosome 11 in Solanum. The I2 homologues in this region form nine well-separated sub-cluster (loci). The sub-loci and their expansion were caused by duplication of individual I2 homologues, since there was no evidence of large (>10 kb) duplications in this region. The duplication frequency of I2 homologues might have varied considerably since different species show a large variation in I2 copy number in different sub-loci. Our data showed that deletions also accounted for part of the I2 locus divergence between different species (Figure 2).

I2 homologues from the centromeric part of the I2 locus were grouped into one clade in the phylogenetic tree of all I2 homologues from Solanum, while homologues from sub-loci 8 and 9 formed four clades (Figure 4). The five clades are equally related with each other, suggesting that they were differentiated at similar time. After differentiation, the homologues from the centromeric part of the I2 locus duplicated and generated the sub-loci 1–7.

#### The structure of I2 locus from the Ty-2 haplotype and S. habrochaites LA1777

The resistance gene Ty-2 against TYLCV in cultivated tomato was introgressed from S. habrochaites accession “B6013” [34]. The recombination at the I2 locus of the Ty-2 haplotype was shown to be highly suppressed [58]. Recombination suppression is possibly caused by inversion (or other dramatic chromosome change) in this region that may prevent pairing between homologous chromosomes. It remains unclear if such chromosome change also exists in any genotypes susceptible to TYLCV. If such susceptible genotypes are identified, they can be crossed with the Ty-2 haplotype and the resistance trait can be fine mapped.

Four accessions of S. habrochaites were included in this study for analysis of I2 homologues. The number of I2 homologues and their sequences seem to vary considerably between different accessions, showing large diversity at this locus. One of the four accessions, accession LA1777, was resistant against strain TYLCV-Cyprus [59] and tolerance to TYLCV strains from Sardinia and Senegal [52,60,61]. Different copy numbers of I2 homologues in the T haplotype (8) and accession LA1777 (13), and no highly similar pairs of genes in the two haplotypes (Figure 3), suggest that they have different I2 locus, consistent with a previous report that the TYLCV...
resistance in LA1777 is not encoded by Ty-2 but by several recessive loci [61].

Sequence exchanges among I2 homologues
All I2 homologues from sub-loci 1–7 in potato were evenly related with each other, similar to the feature of Type I R-genes. However, unlike Type I R-genes, these homologues showed infrequent sequence exchanges, and they are not extensive chimeras though occasional sequence exchanges were detected among them. The presence of a large number of divergent but evenly related homologues and infrequent sequence exchanges suggested that the I2 homologues from sub-loci 1–7 of the I2 locus in potato are Type I R-genes. They are mingled with tomato I2 homologues from sub-loci 1–7 in phylogenetic tree (Figure 4), suggesting that this lineage of Type I genes in potato had existed before the divergence of tomato and potato 7 MYA [53,54], showed that the evolutionary pattern of R-genes may change dramatically within a relatively short evolutionary time.

I2 homologues from sub-locus 9 of potato were grouped into two different clades (Figure 4). Furthermore, the homologues from these two clades are interwoven at sub-locus 9. Members of different lineages of the R1 resistance gene are also interwoven in the R1 resistance gene cluster in potato similar to the organization of the R1 resistance gene family in potato [62]. The interweaving organization of members from different clades suggests that physical proximity is not the main factor for the differentiation of Type I and Type II R-genes [63].

MiRNAs targeting I2 homologues
R-genes, though critical for the survival of plants, may pose threats to plant fitness if accumulated to a high copy number [29]. To reduce their fitness cost, R-genes are often kept at low level of expression. One mechanism for down-regulating the expression of R-genes is through miRNAs, which were recently shown to cleave R-genes and generate tasiRNAs [33]. In addition to the miR482 family targeting I2 homologues in potato [33], we identified and confirmed that miR6024 targets I2 family in tomato. Search of the tomato degradome confirmed the cleavage function of miR6024 and also suggest the cleavage function of tasiRNAs triggered by

Figure 6 Alignment of the miR6024 genes in Solanaceae. The bottom line is consensus sequence. Underlines in the consensus sequences represent miR6024, while dash lines under the consensus sequence represent miR6024*. Dots mean nucleotides identical to that of consensus sequence. Dash lines in sequences represent deletions. Sequence name HZ represents tomato Heinz 1706. DM for potato cultivar DM, CA for C. annuum, NTA for N. tobaccum, NT for N. tomentosiformis, NB for N. benthemiana and NS for N. sylvestris.
miRNAs (see Results section). The miR482 family has high expression level in three tissues of potato (leaf, flower and stolon) (five members, with an average of 35,760 reads per million), while miR6024 family has a much lower expression in tomato (leaf, flower and fruit) (2,540 reads per million). MiR6024 was found in distantly related genus (Nicotiana and Solanum) in Solanaeceae, and therefore it was most likely originated in the common ancestor of Solanaeceae. MiR482 was shown to be an ancient miRNA [64]. However, no cleavage of I2 homologues by miR482 was confirmed in tomato though the miR482 does exist in tomato genome. Therefore, the collective regulation by miR482, miR6024 as well as tasiRNAs triggered by them may control the expression level of I2 homologues in Solanaeceae, and the down-regulation of the I2 homologues by these miRNAs may make the expansion of I2 homologues less costly in fitness. Though silenced by miRNAs, these R-genes can exercise their full resistance function when challenged by pathogens such as viruses and fungi, which can suppress the silencing machinery of host plants [65,66].

Conclusions

Comparative analysis of the I2 locus in tomato and potato showed that the I2 locus contains a large number of homologues with high divergence. Its evolutionary patterns varied considerably between tomato and potato. The I2 family was targeted by at least two miRNAs, which may play important roles in down-regulation and evolution of this resistance gene family.

Availability of supporting data

Sequence data from this study can be found in the GenBank data libraries under accession numbers KJ652840 - KJ652901.

Additional files

Additional file 1: Table S1. I2 homologues used in this study. Table S1-1. I2 homologues identified in the sequenced tomato genome (version 2.40). Table S1-2. I2 homologues identified in the sequenced potato genome (version 3.2.1.11). Table S1-3. I2 homologues from Solanum used in this study. Table S2. Sequence exchanges between I2 homologues. Table S2-1. Sequence exchanges detected between tomato I2 homologues from different clades in Figure 3. Table S2-2. Sequence exchanges detected between potato I2 homologues from different clades in Figure 4.

Additional file 2: Figure S1. Dot plot analysis of the I2 locus in tomato. Figure S2. Dot plot analysis of the I2 locus in potato. Figure S3. Distance tree of I2 homologues from tomato, potato, pepper and tobacco. Figure S4. A sequence exchange tract of 162 bp between a Type I and a Type II I2 homologues. Figure S5. The cleavage site of miR6024 obtained through sequencing RACE-PCR products. Figure S6. A. Alignment of T-I2-3 sequence with a cDNA sequence (T-I2-3) found from a tomato degradome database. B. The abundance of U-derived sRNAs. C. Cleavage sites of two tasiRNAs triggered by miR6024. The bottom lines of each panel represent 5' sequences obtained using RACE-PCR. The line in gene T-I2-3 shows the 3rd tasiRNA, and the line in gene T-I2-6 shows the 5th tasiRNA. D. A degraded RNA retrieved from a degradome database.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CW carried out the molecular and genetic studies, participated in analysis and interpretation of the data and drafted the manuscript. FL helped in the miRNA study and revised the manuscript. HK helped in the evolution study. JC designed the study and revised the manuscript. All authors read and approved the final manuscript.

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