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Intron gain by tandem genomic duplication: a novel case and a modification of the traditional model

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Origin and subsequent accumulation of spliceosomal introns are prominent events in the evolution of eukaryotic gene structure. Recently gained introns would be especially useful for the study of the mechanism(s) of intron gain because the evolutionary traces might have not been erased by randomly accumulated mutations. However, the mechanism(s) of intron gain remain unclear due to the presence of a few solid cases. A widely cited model of intron gain is tandem genomic duplication, in which the duplication of an AGGT-containing exonic segment provides the GT and AG splicing sites for the new intron. However, successful recognition and splicing of an intron require many more signals than those at the two splicing sites. We found that the second intron of the potato RNA-dependent RNA polymerase gene *PGSC0003DMG402000361* is absent in the orthologous genes of other Solanaceae plants, and sequence similarity showed that the major part of the new intron is a direct duplication of the 3' side of the upstream intron. In addition to the new intron, a downstream exonic segment of 168bp has also been duplicated. Most of the splicing signals were inherited from the parental intron/exon structure, including a putative branch site, the polypyrimidine tract, the 3' splicing site, two putative exonic splicing enhancers and the GC contents differentiated between the intron and exon. We propose a modified version of the tandem genomic duplication model, termed as the partial duplication of the preexisting intron/exon structure.

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Intron gain by tandem genomic duplication: a novel case and a modification of the traditional model

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ABSTRACT

Origin and subsequent accumulation of spliceosomal introns are prominent events in the evolution of eukaryotic gene structure. Recently gained introns would be especially useful for the study of the mechanism(s) of intron gain because the evolutionary traces might have not been erased by randomly accumulated mutations. However, the mechanism(s) of intron gain remain unclear due to the presence of a few solid cases. A widely cited model of intron gain is tandem genomic duplication, in which the duplication of an AGGT-containing exonic segment provides the GT and AG splicing sites for the new intron. However, successful recognition and splicing of an intron require many more signals than those at the two splicing sites. We found that the second intron of the potato RNA-dependent RNA polymerase gene *PGSC0003DMG402000361* is absent in the orthologous genes of other Solanaceae plants, and sequence similarity showed that the major part of the new intron is a direct duplication of the 3′ side of the upstream intron. In addition to the new intron, a downstream exonic segment of 168bp has also been duplicated. Most of the splicing signals were inherited from the parental intron/exon structure, including a putative branch site, the polypyrimidine tract, the 3′ splicing site, two putative exonic splicing enhancers and the GC contents differentiated between the intron and exon. We propose a modified version of the tandem genomic duplication model, termed as the partial duplication of the preexisting intron/exon structure.

INTRODUCTION

Although, spliceosomal introns are the characteristic feature of eukaryotic nuclear genes, their origin and subsequent accumulation during evolution remain obscure. There are several purposed models of the spliceosomal introns gain, which include intron transposition, transposon insertion, tandem genomic duplication, insertion of an exogenous sequence during double-strand-break repair, insertion of a group II intron, intron transfer and intronization (Yenerall & Zhou 2012). Comparative analyses of discordant intron positions among conserved homologous genes have been carried out in diverse eukaryotic lineages. Except for a few studies (Fablet et al. 2009; Li et al. 2009; Torriani et al. 2011; van der Burgt et al. 2012; Verhelst et al. 2013), the observed frequency of intron gain has generally been found to be much lower than those of the intron loss, and there is very limited supporting evidence for the intron gain models (Csuros et al. 2011; Hooks et al. 2014; Irimia & Roy 2014; Roy & Gilbert 2005; Roy & Penny 2006; Yenerall et al. 2011; Yenerall & Zhou 2012; Zhu & Niu 2013). Recently, Collemare et al. (2013) claimed that the abundance of introns in extant eukaryotic genomes could not be explained by traditional models of intron gain, but can be possible by a new model, the insertion of introner-like elements (van der Burgt et al. 2012). Here, we investigate a novel case of intron gain and also highlight the importance of tandem genomic duplications in gene evolution.

MATERIALS AND METHODS

The genome sequences and annotation files of potato (*Solanum tuberosum*) (PGSC_DM_v3), tomato (*Solanum lycopersicum*) (ITAG2.3), and tobacco (*Nicotiana benthamiana*) (version 0.4.4) were downloaded from SGN (Sol Genomics Network) (Bombarely et al. 2011), while Pepper Genome Database (release 2.0) (Qin et al. 2014) was used for the pepper (*Capsicum annuum* L.)

(Zunla-1). The scaffold sequences of eggplant (*Solanum melongena*) were downloaded from NCBI (SME_r2.5.1, http://www.ncbi.nlm.nih.gov/genome/). The SAR files of the potato whole-genome shotgun (WGS) reads (SRP007439) and the leaf, tuber, and mixed-tissue transcriptomes (SRP022916, SRP005965, SRP040682, and ERP003480) were retrieved from the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra/). We mapped the RNA-Seq reads to the genomes by using TopHat version 2.0.8 (Kim et al. 2013), while BWA (alignment via Burrows-Wheeler transformation, version 0.5.7) (Li & Durbin 2009) was used for the WGS reads. We used default parameters for both programs. The orthologous proteins were identified by using the best reciprocal BLAST hits with a threshold E value of $< 10^{-10}$. In addition, the orthologous relationships were confirmed by using the SynMap (http://genomevolution.org/CoGe/SynMap.pl).

The RNA-dependent RNA polymerase (*RdRp*) genes encode those enzymes which catalyze the replication of RNA from an RNA template. They have been identified in all the major eukaryotic groups and play crucial roles in the regulation of development, maintenance of genome integrity, and defense against the foreign nucleic acids (Willmann et al. 2011; Zong et al. 2009). The *PGSC0003DMG402000361* orthologous sequence in eggplants was manually annotated with references to the annotations of the orthologous genes in potato, tomato, pepper, and tobacco.

By aligning 9,883 groups of orthologous mRNAs among potato, tomato and pepper, we obtained 34,364 conserved introns in potatoes with length > 60 bp. Among these conserved introns, we searched the consensus sequences of the 5′ splicing sites, the branch sites, the polypyrimidine tracts, and the 3′ splicing sites according to Irimia and Roy (2008) and Schwartz, et al. (2008). The information content of these sites was calculated by using the WebLogo 3.4

online (http://weblogo.threeplusone.com/create.cgi) (Crooks et al. 2004). The exonic splicing enhancers of *A. thaliana* were identified by Pertea et al. (2007). We used them as query and found 50 bp exonic sequence downstream of the target intron.

RESULTS AND DISCUSSION

By comparing the orthologous genes of tomatoes (*Solanum lycopersicum*), potatoes (*Solanum tuberosum*) and other Solanaceae plants, we found 11 cases of precise intron loss and six cases of imprecise intron loss (Ma et al. 2015). At the same time, we found the sign of an imprecise intron gain in potato gene, *PGSC0003DMG402000361*. The second intron of this gene is found unique in potatoes (Fig. 1). By analyzing the transcriptomic data of potato, we found 109 RNA-Seq reads that are exclusively mapped to the annotated exon-exon boundary (Supplemental Information 1: Table S1), which confirmed the annotation of this intron. Based on the phylogenetic tree of the species being compared (Fig. 2), there were two possible explanations for the presence/absence of the intron. The first was the gain of a new intron in potatoes, and the second was four parallel intron loss events occurred in the other four species: tomato, eggplant, pepper, and tobacco. According to the principle of parsimony, we concluded that the second intron of the potato gene *PGSC0003DMG402000361* was gained after the divergence of potatoes and tomatoes.

The intron gain was accompanied by a 168 bp insertion in the downstream exon (Fig. 1). For BLAT search (Kent 2002), new intron and the inserted exonic sequence was used as a query sequence against the whole potato genome, and it was found that the combined sequence is a direct duplication of the upstream sequence that co-occurs with a small insertion of an exogenous sequence (10 bp) between the duplicates (Fig. 3A). We were aware of this fact that two nearly identical regions in a reference genome might either be a true duplicate or a false due to an error

in genome assembly. To verify the duplication in the potato gene *PGSC0003DMG402000361*, we found three sources of evidence. Firstly, 62 whole genome shotgun (WGS) reads were exclusively mapped crossing the four boundaries of two duplicates (Supplemental Information 1: Table S2). Secondly, 109 RNA-Seq reads were exclusively mapped crossing the boundary of the two duplicates in mature mRNA, *i.e.,* the position of the new intron (Supplemental Information 1: Table S1). Thirdly, there are ten nucleotide differences between the duplicates (Fig. 3B).

Close examination of the coding region confirmed that the duplication and the insertion did not cause any frame-shifts. Furthermore, we tested whether *PGSC0003DMG402000361* is still a functional gene, or a pseudogene, by surveying its nonsynonymous and synonymous substitution rates, d_N and d_S, respectively. Using the phylogenetic tree of *PGSC0003DMG402000361* and its orthologous genes in tomato, pepper, and tobacco, we performed a likelihood-ratio test (LRT) to compare two models. The first model was the null hypothesis that the gene was undergoing neutral evolution, in which case the d_N/d_S value of *PGSC0003DMG402000361* would be equal to one. In the alternative model, the estimated value of d_N/d_S would be < 1 (Yang 2007). The d_N/d_S was 0.3101; the LRT statistic, $2\Delta\ell$ (twice the log likelihood difference between the two compared models), was 74.7; and the χ^2 test supported the hypothesis that the *PGSC0003DMG402000361* gene was subject to purifying selection ($P < 10^{-16}$).

According to Logsdon et al. (1998), strong evidence of intron gain must satisfy the two conditions. The first one is a clear phylogeny to provide support for the intron gain, while the second is an identified source element of the gained intron. Given the clear phylogeny and the identity of the source sequence, we consider the second intron of the potato gene *PGSC0003DMG402000361* to be a well-supported case of a newly gained intron.

125 The present case of intron gain is somewhat different from the tandem genomic duplication
126 model of intron gain that was originally put forward by Rogers (1989). In that model, tandem
127 duplication of an exonic segment harboring the AGGT sequence generates two splice sites for
128 the new intron: 5′-GT and 3′-AG, and the new intron comes from the duplication of exonic
129 sequence. It is now well known that the two splice sites do not contain sufficient information to
130 unequivocally determine the exon-intron boundaries (Lim & Burge 2001). Accurate recognition
131 and efficient splicing of an intron also requires a polypyrimidine tract, an adenine nucleotide at
132 the branch site, and many other *cis*-acting regulatory motifs (Schwartz et al. 2009; Spies et al.
133 2009; Wang & Burge 2008; Wang et al. 2004). Duplication of exonic segments might happen to
134 generate a combination of the required signals and also produce a functional intron (Hellsten et
135 al. 2011), but it is unlikely that preexisting polypyrimidine tracts and other splicing signals are
136 commonly present in coding sequences. Additionally, introns are often remarkably richer in AU
137 than exons (Amit et al. 2012), and this difference has been demonstrated to be a requirement for
138 efficient splicing (Carle-Urioste et al. 1997; Luehrsen & Walbot 1994). The phenomena of direct
139 introns gain from duplicated exonic segments is particularly unlikely in plants, and it is due to
140 the striking difference of base content between exons and introns. In the present case of intron
141 gain, the duplication includes the 3′ side sequence of an intron and the 5′ side of the downstream
142 exon (Fig. 3A). The 3′ splicing site signal (CAG), the polypyrimidine tract
143 (TCTTCCAATGCCT), and the putative branch site (TTTAC) of this novel intron was inherited
144 from the parental intron (Fig. 3B, 3C). Moreover, the two overlapped putative exonic splicing
145 enhancers of the 3′ flanking exon, TCAGCT and CAGCTC, and the GC contents differentiated
146 between the intron and exon (36% *vs.* 46%) were also inherited from the parental copy. The 5′
147 splicing signal of the novel intron, GTAAG, was provided by the exogenous sequence of 10 bp.

Utilization of some of the active splicing signals of the parental intron is apparently a more efficient method of intron gain. Therefore, we propose a modified version of the tandem genomic duplication model, termed as partial duplication of a preexisting intron and the flanking exon. Segmental duplication containing entire introns would be more likely to increase the gene intron number and also has been observed previously (Gao & Lynch 2009). In the present paper, we confine our discussion to the creation of new introns rather than the propagation of preexisting introns.

The modified version of the tandem genomic duplication model of intron gain could also be termed as imprecise intron gain, which highlights the co-occurring insertion of the coding sequence. Generally, the researchers seek intron gains in highly conserved orthologous genes. Thus, only introns flanking conserved exonic sequences are likely to be identified as a new one. Due to this methodology, the frequency of intron gain by segmental duplication might have been underestimated previously. To be consistent with this idea, a study that specifically explored intron gains by segmental duplications revealed tens of new introns in humans, mice, and *Arabidopsis thaliana* (Gao & Lynch 2009). This result is in stark contrast to the comparative studies of their highly conserved orthologous genes, which found very few or no intron gains at all (Coulombe-Huntington & Majewski 2007; Fawcett et al. 2012; Roy et al. 2003; Yang et al. 2013). Considering the high frequency of internal gene duplications, which is 0.001–0.013 duplications/gene per million years (Gao & Lynch 2009), it can be stated that intron gain by segmental duplication may be an important force shaping the eukaryotic gene structure. With the increasing number of very closely related genomes (*i.e.*, diverged within ten million years) to be sequenced, we expect to find more intron gains by segmental duplication in the near future.

CONCLUSIONS

In the potato gene *PGSC0003DMG402000361*, we found a novel intron originated from tandem duplication. The duplicate includes the 3′ side sequence of an intron and the 5′ side of the downstream exon. Most splicing signals which include, a putative branch site, the polypyrimidine tract, the 3′ splicing site, two putative exonic splicing enhancers and the GC contents differentiated between the intron and exon were inherited from the parental intron/exon structure. By contrast, the widely cited model of intron gain is tandem duplication of an exonic segment containing AGGT, which would create the GT and AG splicing sites. The case of intron gain which we observed, requires a modified version of the tandem genomic duplication model: partial duplication of the preexisting intron/exon structure. As we see, this modified version is more consistent with the mechanisms of intron recognition and splicing (Schwartz et al. 2009; Spies et al. 2009; Wang & Burge 2008; Wang et al. 2004).

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318 Figures

319 **Figure 1. Alignments of coding sequences showing the intron gain and a flanking insertion**
320 **in the potato gene *PGSC0003DMG402000361*.**

321 The presence and absence of the intron are represented by 1 and 0, respectively. The orthologous
322 genes used as references are *Solyc12g008410.1* in tomato, *Capana09g000243* in pepper, and
323 *NbS00003153g0003* in tobacco. The orthologous region in eggplants was manually identified by
324 the best reciprocal program, BLAST, and manually annotated.

325

326 **Figure 2. Phylogenetic tree used to identify the intron gain in *Solanum tuberosum*.**

327 The tree was adapted from Särkinen et al. (2013) and is not scaled according to substitution rates.
328 The presence and absence of the intron are represented by + and –, respectively.

329

330 **Figure 3. The intron gain by tandem genomic duplication in the potato gene**
331 ***PGSC0003DMG402000361*.**

332 **(A)** A schematic diagram showing the creation of a new intron by partial duplication of the
333 parental intron (marked in blue line) and the insertion of a short exogenous sequence (marked in
334 red line). **(B)** Alignment of the two copies of the duplication and the inserted exogenous
335 sequence (marked in red). The splicing sites, the putative branch site, the polypyrimidine tract,
336 and two overlapping putative exonic splicing enhancers (ESE; TCAGCT and CAGCTC) are
337 underlined. Sites differing between the two copies are indicated with bold blue letters. **(C)** The

consensus sequences of the introns conserved among potatoes, tomatoes and peppers. These sequences were used to recognize the splicing signals for the new intron.

Figure 1(on next page)

Alignments of coding sequences showing the intron gain and a flanking insertion in the potato gene *PGSC0003DMG402000361*

The presence and absence of the intron are represented by 1 and 0, respectively. The orthologous genes used as references are *Solyc12g008410.1* in tomato, *Capana09g000243* in pepper, and *NbS00003153g0003* in tobacco. The orthologous regions in eggplants were manually identified by the best reciprocal program, BLAST, and manually annotated.

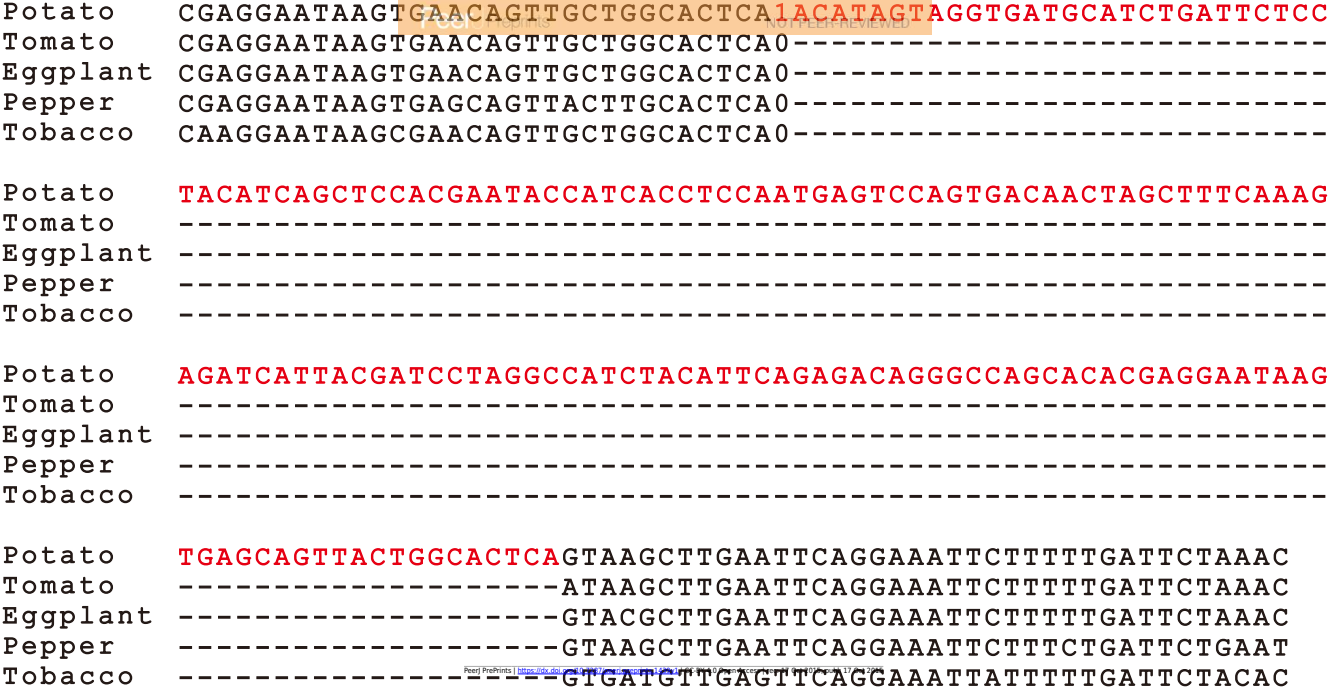

Potato CGAGGAATAAGTGAACAGTTGCTGGCACTCA1ACATAGTAGGTGATGCATCTGATTCTCC
Tomato CGAGGAATAAGTGAACAGTTGCTGGCACTCA0----------
Eggplant CGAGGAATAAGTGAACAGTTGCTGGCACTCA0----------
Pepper CGAGGAATAAGTGAGCAGTTACTTGCACTCA0----------
Tobacco CAAGGAATAAGCGAACAGTTGCTGGCACTCA0----------
Potato TACATCAGCTCCACGAATACCATCACCTCCAATGAGTCCAGTGACAACTAGCTTTCAAAG
Tomato ----------
Eggplant ----------
Pepper ----------
Tobacco ----------
Potato AGATCATTACGATCCTAGGCCATCTACATTCAGAGACAGGGCCAGCACACGAGGAATAAG
Tomato ----------
Eggplant ----------
Pepper ----------
Tobacco ----------
Potato TGAGCAGTTACTGGCACTCAGTAAGCTTGAATTCAGGAAATTCTTTTTGATTCTAAAC
Tomato ----------ATAAGCTTGAATTCAGGAAATTCTTTTTGATTCTAAAC
Eggplant ----------GTACGCTTGAATTCAGGAAATTCTTTTTGATTCTAAAC
Pepper ----------GTAAGCTTGAATTCAGGAAATTCTTTCTGATTCTGAAT
Tobacco ----------GTGATGTTGAGTTCAGGAAATTATTTTTGATTCTACAC

Figure 2 (on next page)

Phylogenetic tree used to identify the intron gain in ***Solanum tuberosum***

The tree was adapted from Särkinen et al. (2013) and is not scaled according to substitution rates. The presence and absence of the intron are represented by + and - , respectively.

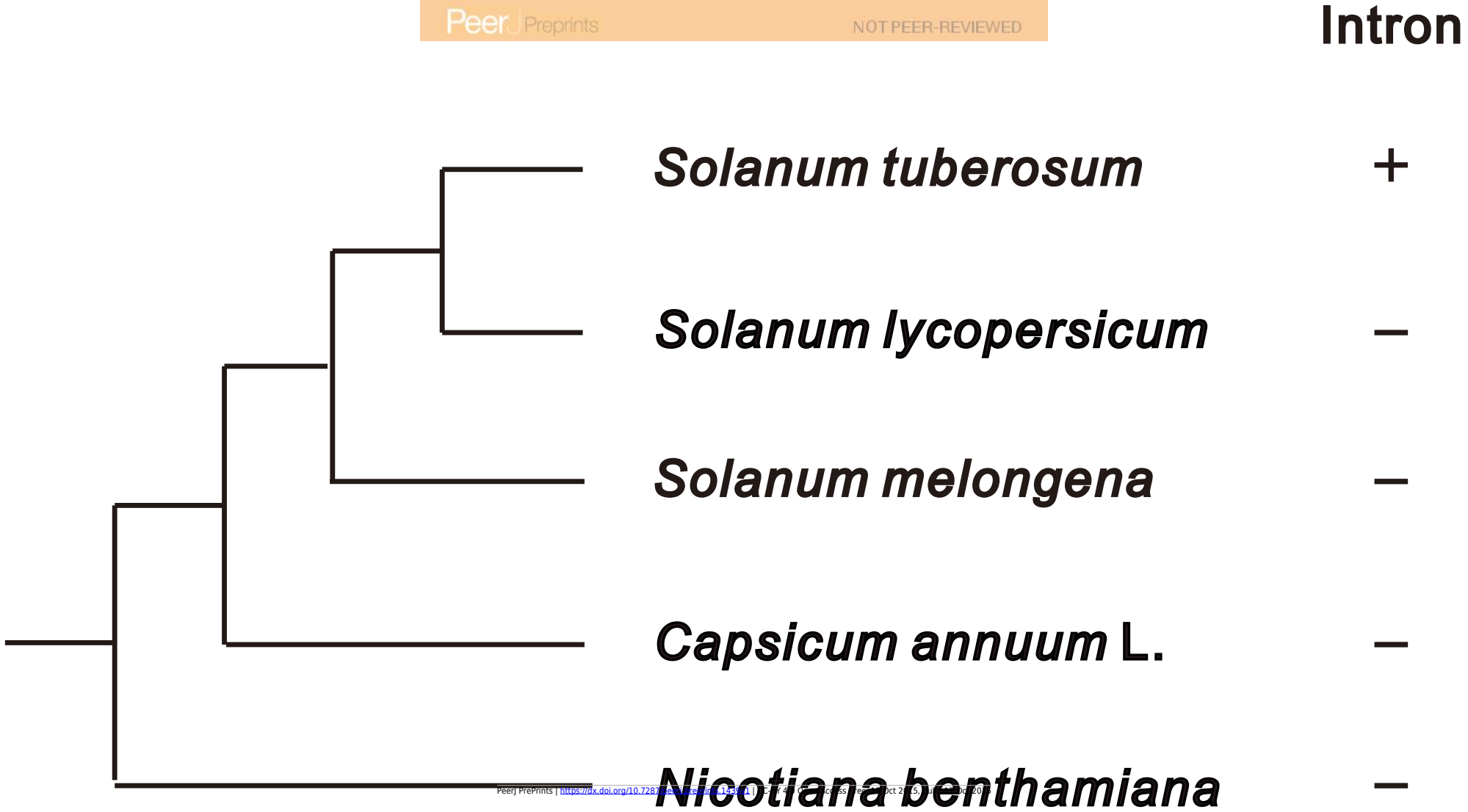
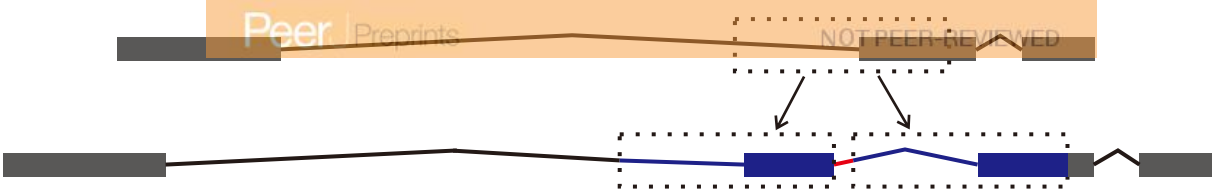


Figure 3(on next page)

The intron gain by tandemgenomic duplication in the potato gene ***PGSC0003DMG402000361***

(A) A schematic diagram showing the creation of a new intron by partial duplication of the parental intron (marked in blue line) and the insertion of a short exogenous sequence (marked in red line). **(B)** Alignment of the two copies of the duplication and the inserted exogenous sequence (marked in red). The splicing sites, the putative branch site, the polypyrimidine tract, and two overlapping putative exonic splicing enhancers (ESE; TCAGCT and CAGCTC) are underlined. Sites differing between the two copies are indicated with bold blue letters. **(C)** The consensus sequences of the introns conserved among potatoes, tomatoes and peppers. These sequences were used to recognize the splicing signals for the new intron.

A



B

```
Downstream gtaagcttgaattagtttaggcttaatgaagaacttgttcaatttttttattggttgcga
Upstream   ----------attagtttaggcttaatgaagaacttgttcaaatttttttattggttgcga

Downstream tctcttcttcc-ttttttttgcatatttacaactctacatgtaaactatgttgctcggact
Upstream   tctcttcttcctttttttttgcatatttacaactctacatgtaaactatgttgctcggact

Downstream ctcaaaaactgttgaacccgtgttggattctccaaaatgcactacttttggagtattcga
Upstream   ctcaaaaactgttgaacccgtgttggattctccaaaatgcactacttttggagtattcga

Downstream tacacacttttgaagagtccgaacaacacaacatgt-aatgtactcagacctttcaagaa
Upstream   tacacacttttgaagagtctgaacaacacaacatgtaaacatactcagacctttcaaaaa

Downstream ttctagtttaccaatgatggtcttccaatgcctgcagACATAGTAGGTGATGCATCTGAT
Upstream   ttctagtttaccaatgatggtcttccaatgcctacagACATAGTAGGTGATGCATCTGAT

Downstream TCTCCTACATCAGCTCCACGAATACCATCACCTCCAATGAGTCCAGTGACAACTAGCTTT
Upstream   TCTCCTACATCAGCTCCACGAATACCATCACCTCCAATGAGTCCAGTGACAACTAGCTTT

Downstream CAAAGAGATCATTACGATCCTAGGCCATCTACATTCAGAGACAGGGCCAGCACACGAGGA
Upstream   CAAAGAGATCATTACGATCCTAGGCCATCTACATTCAGAGACAGGGCCAGCACACGAGGA

Downstream ATAAGTGAGCAGTTACTGGCACTCA
Upstream   ATAAGTGAACAGTTGCTGGCACTCA
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C