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# Structural and phylogenetic analysis of *Pto*-type disease resistance gene candidates in *Hevea brasiliensis*

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**Abstract**: The tomato *Pto* gene encodes a serine/threonine kinase (STK) whose molecular characterization provides valuable insights into the disease resistance mechanism of tomato and it is considered as a promising candidate for engineering broad-spectrum pathogen resistance in this crop. In this study, a pair of degenerate primers based on conserved subdomains of plant STKs similar to the tomato Pto protein were used to amplify similar sequences in *Hevea brasiliensis*. A fragment of ~550 bp was amplified, cloned and sequenced. The sequence analysis of several clones revealed twelve distinct sequences were highly similar to STKs. Based on their significant similarity with the tomato Pto protein (BLASTX *E* value <3e-53), seven of them were classified as *Pto* resistance gene candidates (*Pto*-RGCs). Multiple sequence alignment of the hevea *Pto*-RGC products revealed that these sequences contained several conserved subdomains in most STKs and also several conserved residues that are crucial for *Pto* function. Moreover, the phylogenetic analysis showed that the hevea *Pto*-RGCs were clustered with *Pto* suggesting a common evolutionary origin with this *R* gene. The *Pto*-RGCs isolated in this study represent a valuable sequence resource that could assist in the development of disease resistance in hevea.

**Key words**: disease resistance gene, *Pto*, serine/threonine kinase, *Hevea brasiliensis*

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# 巴西橡胶 *Pto* 类抗病同源序列的克隆与系统发育重建

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**摘 要**: 番茄 *Pto* 基因是一类可以编码丝氨酸/苏氨酸激酶(STK)序列的广谱抗性候选基因,其序列克隆与鉴定为深入了解番茄的抗病机制奠定了基础。在该研究中,一对依据 *Pto* 基因的保守序列设计的简并引物被用来扩增巴西橡胶中 *Pto* 基因抗病同源序列,扩增得到了一个约 550 bp 的基因片段,其随后被克隆并测序。序列分析发现,其中的 7 个抗病同源序列与 *Pto* 基因高度同源(BLASTX *E* value <3e-53),所以其被认为是 *Pto* 基因抗病同源序列(*Pto*-RGCs)。通过巴西橡胶的 *Pto*-RGCs 多序列比对表明,这些序列包含了多个 STKs 保守的次级结构

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域。此外,系统发育分析也表明,巴西橡胶的 *Pto*-RGCs 属于 *Pto* 基因同源的 *R* 基因。该研究结果中 *Pto*-RGCs 可为巴西橡胶抗病的发展提供一个有效的基因资源。

**关键词**: 抗病基因, *Pto*, 苏氨酸/丝氨酸激酶, 巴西橡胶

Natural rubber is not only an important industrial material but also an important strategic resource, the Para rubber tree (*Hevea brasiliensis*), is the only commercial source at present, due to its high rubber content and quality (Backhaus, 1985). Natural rubber production is currently threatened by a wide spectrum of pathogens including viruses, bacteria, fungi and nematodes. They reduce yield, affect the quality, debilitate or kill the host plant (Jacob et al, 1989; Lespinasse et al, 2000). Currently, the ascomycete fungus *Microcyclus ulei* (South American leaf blight, SALB) which was originated from South America, have become the most important disease of there, even destruction some rubber plantation there, more worse there is no evidence that there is an absolute effective chemical control exist (Alencar et al, 1975; Albuquerque et al, 1987). Genetic resistance is the most suitable strategy to control these pathogens in the field and there are sources of resistance to both of them in wild clones germplasm. However, breeding for pathogen resistance in hevea is limited by the long life cycle, trip-loidy and sterility in most commercial cultivars. Molecular biotechnology has the potential to overcome these constraints by transferring single or even multiple disease resistance (*R*) genes into the genome of susceptible hevea cultivars using either biolistics or agrobacterium-mediated transformation. Although transformation technologies have been developed for hevea in various laboratories around the world, no hevea *R* gene has been isolated to date (Huang et al, 2010).

The tomato *Pto* gene is one of the best-characterized and most intensively studied *R* genes (Pedley & Martin, 2003). *Pto* confers hypersensitive response-mediated resistance against strains of *Pseudomonas syringae* pv. *tomato* that express the avirulence proteins AvrPto or Avr-PtoB (Martin et al, 1993; Kim et al, 2002). Overexpression of *Pto* in tomato under the control of the strong cauliflower mosaic virus (CaMV) 35S promoter has been shown to activate defense responses in the absence of pathogen inoculation. *Pto*-overexpressing plants show resistance not only to *P. syringae* pv *tomato* but also to *Xanthomonas campestris* pv. *vesicatoria* and to the fungal pathogen *Cladosporium fulvum* (Tang et al, 1999). With the Bioinformatics analysis software, the *Pto* encodes a cytoplasmic serine/threonine protein kinase (STK) and also with several conserved subdomains, southern hybridization using the tomato *Pto* gene as probe revealed the presence of *Pto*-RGCs in many plant species such as *Arabidopsis*, bean, soybean, pea, rice, maize, barley, wheat and sugarcane (Martin et al, 1993). *Pto*-type disease resistance gene analogues had successfully amplified from banana, potato, bean, grapevine etc. (Vleeshouwers et al, 2001; Vallad et al, 2001; Digaspero & Cipriani, 2003), but since no molecular characterization of hevea *Pto*-RGCs has been published, the objectives of this study were to obtain *Pto*-RGCs from hevea using degenerate PCR and to determine the structure and phylogenetic relationships of the hevea *Pto*-RGCs.

## 1 Materials and Methods

### 1.1 Plant material and DNA extraction

The hevea wild germplasm 'XJ000072' was chosen for PCR amplification of *Pto*-RGC sequences because it shows resistance to a range of hevea pathogens, including the most destructive such as *Microcyclus ulei* and *Oidium heveae* . The genomic DNA was isolated using QIAGEN DNeasy Plant Minikit(QIAGEN Inc., Valencia, CA)according to manufacturer's instructions.

### 1.2 Degenerate PCR

A pair of degenerate primers designed by Vallad et al, (2001), forward 5′-TNGGNSANGGNGKNTTYGG-3′ and reverse 5′-ACNCCRAANGARTANACRTC-3′, was used to amplify the region between the subdomains I and IX of STKs. The degenerate PCR reaction was performed in a 50 μL reaction volume containing 300 μmol · $L^{-1}$ of dNTPs, 4 μmol · $L^{-1}$ of each degenerate primer forward

and reverses, 1 U of Taq DNA polymerase (Invitrogen™), 1 × PCR buffer, 1.5 mmol · $L^{-1}$ $MgCl_2$ and approximately 200 ng of genomic DNA. PCR conditions were 95 ℃ for 3 min, followed by 35 cycles of 95 ℃ for 30 s, 45 ℃ for 30 s and 72 ℃ for 1 min; and an additional 10 min extension at 72 ℃ was included.

### 1.3 Cloning and sequencing

PCR products were visualized on a 1% agarose gel stained with ethidium bromide. A band of the expected size was excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. Purified PCR products were cloned into the pGEM©-T easy plasmid vector (Invitrogen Corp., Carlsbad, CA). Plasmids were transferred by electroporation into *Escherichia coli* DH 5α competent cells. Bacteria were plated onto LB medium containing ampicillin, X-Gal and IPTG, and recombinant plasmids were chosen by blue/white selection (Sambrook & Russell, 2001). Plasmid DNA was purified by the alkaline lysis method (Sambrook & Russell, 2001) and sequenced using the BigDye terminator sequencing kit version 3.1 (Applied Biosystems) according to manufacturer's instructions. The sequencing products were separated with an ABI 3 730 automatic sequencer (Applied Biosystems) through the capillary separation service of the Chinese academy of agricultural science. Selected clones were sequenced in both orientations.

### 1.4 Sequence edition, similarity searches and multiple-sequence alignment

All sequences were assembled and edited using the programs SEQMAN and EDIT, respectively of the Lasergene software package version 4.03 (DNASTAR, Madison, WI, USA). The degenerate primer sequences were removed from each sequenced clone so only the region between the end of subdomain I and the start of subdomain IX of STKs was considered for further analysis. Predicted amino acid sequences were generated using the translate tool of the EDIT program (Lasergene software). Similarity searches were conducted with the BLASTX program (Altschul et al, 1997) through the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov) using the default settings. Percent amino acid identity between predicted protein sequences was determined with the MEGALIGN program of the Lasergene software using the default settings. Determination of conserved amino acids in hevea *Pto*-RGC sequences was carried out with the programs Clustal X version 2.1 (Larkin et al, 2007) and WebLogo version 3.0 (Crooks et al, 2004) (http://weblogo.berkeley.edu/) using the default settings.

### 1.5 Phylogenetic analysis

Phylogenetic trees were constructed by the neighbor-joining (NJ) method using the NJ algorithm implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.10 with the Poisson correction. Bootstrapping (1 000 replicates) was used to evaluate the degree of support for a particular grouping pattern in the phylogenetic tree. Protein sequences belonging to twelve groups of characterized STKs from *Arabidopsis thaliana* (Hardie,1999), a phosphoenolpyruvate carboxylase kinase (PEPck) (GenBank accession No. AF162660) from *A. thaliana* (Hartwell et al, 1999), the tomato Pto protein (GenBank accession No. A49332) and *Pto*-RGCs from different plant species were retrieved from the GenBank for the phylogenetic tree constructions. The tomato *Pto* disease resistant protein was used as query in BLASTP (Altschul et al, 1997) searches to retrieve amino acid sequences of *Pto*-RGCs from the GenBank. Only the region between the end of subdomain I and the start of subdomain IX was considered for the phylogenetic tree constructions.

## 2 Results and Analysis

### 2.1 Identification of *Pto* resistance gene candidates in hevea

PCR amplification of hevea genomic DNA with a pair of degenerate primers previously used by Vallad et al (2001) generated an expected band of ~550 bp. This band was cloned and a total of 50 clones were sequenced. The primer sequences were removed from each sequenced clone for further analysis. Of the 50 sequenced clones (STK-1 to STK-50), 32 presented uninterrupted open reading frames (ORFs), while the oth-

er eighteen sequences presented multiple stop codons in all reading frames, and as a result they were not further investigated. Similarity searches of the 32 hevea sequences using the BLASTX algorithm (Altschul et al, 1997) against the NCBI non-redundant database revealed significant similarity to known STKs (*E* value < 3e-53), including the disease resistance protein Pto from tomato. A threshold value of 85% amino acid identity previously used by Vallad et al (2001) to classify *Pto*-RGC clones from bean into classes or groups was used in the present study, therefore hevea clones with greater than 85% amino acid identity were considered to be part of the same group.

A total of twelve distinct groups of STK-like sequences were identified, most of which contained redundant or highly similar clones ( > 97% amino acid identity). Seven groups were designated as *Pto* resistance gene candidates (*Pto*-RGCs) based on their significant similarity with the tomato *Pto* disease resistance protein (*E* value < 3e-53). The other five groups showed significant similarity to other types of STKs, which are described later in the text. Each group was designated by the name of a single clone representative of the group and used for further analysis. Percent amino acid identity between the predicted amino acid sequence of *Pto*-RGCs and the corresponding region of the Pto protein ranged from 64.3% (STK40) to 70.9% (STK25) (Table 1), whereas amino acid identity among the *Pto*-RGCs ranged from 70.3% (STK 33 vs. STK 40) to 99.5% (STK3 vs. STK 12)(Table1). BLASTX searches also revealed that no *Pto*-RGCs were highly similar ( > 92% amino acid identity) to hevea sequences present in the GenBank database.

Table 1 Percent amino acid identity between the hevea *Pto*-RGCs and the tomato *Pto* disease resistance protein (Martin et al, 1993)

| *Pto*-RGC[a] | STK3 | STK7 | STK12 | STK25 | STK33 | STK37 | STK40 | Pto |
|---|---|---|---|---|---|---|---|---|
| hSTK3 | – | 78.1 | 99.5 | 83.6 | 77.6 | 83.1 | 77.5 | 70.3 |
| STK7 | … | – | 77.6 | 85.8 | 99.5 | 85.8 | 70.9 | 69.2 |
| STK12 | … | … | – | 84.2 | 77.0 | 83.6 | 76.9 | 69.8 |
| STK25 | … | … | … | – | 85.2 | 98.4 | 74.2 | 70.9 |
| STK33 | … | … | … | … | – | 85.2 | 70.3 | 69.8 |
| STK37 | … | … | … | … | … | – | 73.6 | 70.3 |
| STK40 | … | … | … | … | … | … | – | 64.3 |
| *Pto* | … | … | … | … | … | … | … | – |

## 2.2 Isolation of other hevea serine/threonine kinase-like sequences

The degenerate primers used in this study were designed from the conserved subdomains I and IX of the STKs *Pto*, Fen and Pti1 of tomato, and MHK and APK1 of *Arabidopsis* (Vallad et al, 2001). Therefore, these primers have the potential to isolate not only *Pto*-RGCs but also other types of plant STKs. In agreement with this observation five additional STK-like sequences from hevea were identified in BLASTX searches (Table 2). Four of them (STK8, STK15, STK17 and STK 48) showed significant similarity to the receptor-like kinase (RLK) subfamily (*E* value < 2e-64), whereas the remaining sequence STK43 showed a significant similarity to a receptor-like kinase (*E* value = 3e-123) from *Platanusx acerifolia*.

## 2.3 Multiple sequence alignment and phylogenetic analysis

A multiple sequence alignment using the Clustal X program was performed with the predicted amino acid sequences of the seven hevea *Pto*-RGCs and the corresponding region of the tomato Pto protein (Fig. 1). The alignment revealed that several features of the Pto protein are highly conserved in the hevea *Pto*-RGCs such as the STK subdomains internal to the degenerate primer sequences, the presence of the activation domain between

Table 2　Best BLASTX hits of other hevea serine/threonine kinase-like sequences isolated in this study

| STK-like sequence | GenBank accession number | Plant | Similar to | Length of the BLASTX alignment (in amino acids) | Identity (%) | Similarity (%) | Expect value (*E*) |
|---|---|---|---|---|---|---|---|
| STK8 | ABV30720 | *Prunus avium* | Kinase-like protein | 185 | 74 | 99 | 5e-94 |
| STK15 | ABV30718 | *Prunus avium* | Kinase-like protein | 158 | 77 | 99 | 4e-96 |
| STK17 | ABV30718 | *Prunus avium* | Kinase-like protein | 185 | 77 | 100 | 2e-99 |
| STK43 | AEC04745 | *Platanus x acerifolia* | Receptor-like kinase | 187 | 93 | 100 | 3e-123 |
| STK48 | ABV30720 | *Prunus avium* | Kinase-like protein | 184 | 71 | 99 | 8e-87 |

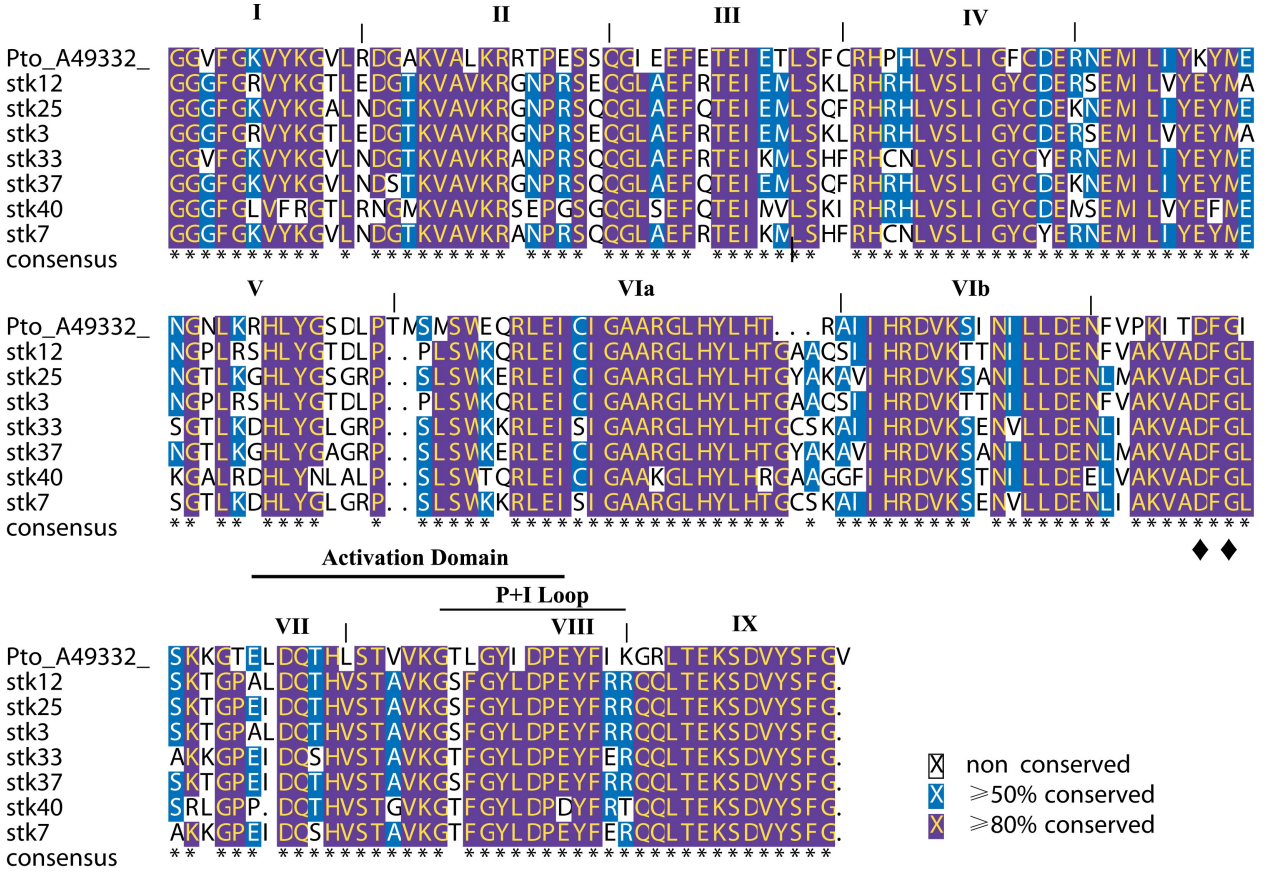


Fig. 1　Clustal X alignment of the deduced amino acid sequences of eleven hevea *Pto*-RGC sequences together with the corresponding region of the Pto protein. The subdomains, activation domain and P + 1 loop of protein kinases (Hanks et al, 1988; Hanks & Quinn, 1991; Vallad et al, 2001) are indicated. Conserved amino acids in plant serine/threonine kinases (Hanks & Quinn, 1991) and *Pto* autophosphorylation sites (Sessa et al, 2000) are indicated with asterisks and grey circles, respectively. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

subdomains Ⅶ and Ⅷ, and its internal P+1 loop site, which is responsible for the specific binding of Avr*Pto* (Frederick et al, 1998), and also several invariant amino acids distributed throughout the sequences. In addition, three of the four autophosphorylation sites (serine or threonine) in the activation domain of *Pto* (Sessa et al, 2000) are conserved in the corresponding region of all hevea *Pto*-RGCs. The alignment also showed that

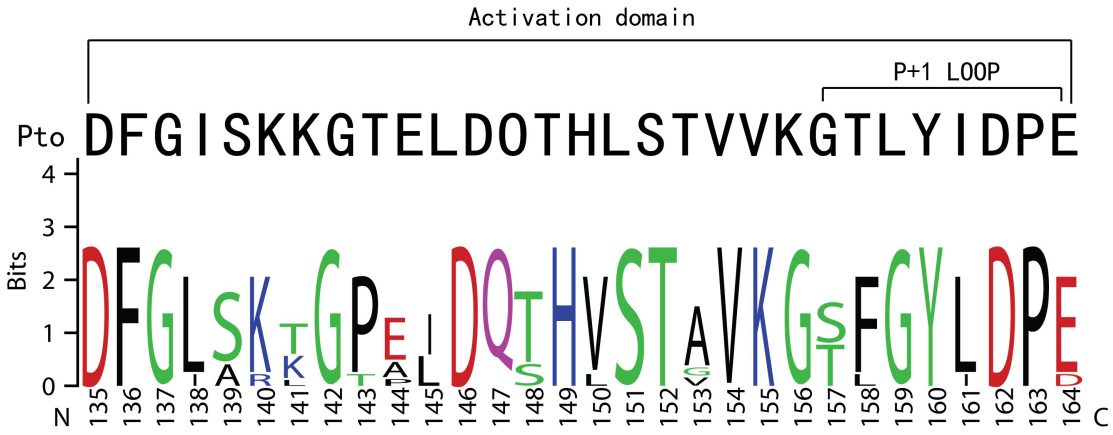


Fig. 2 Comparison of the tomato *Pto* activation domain (30 amino acids in length) with the sequence Logo of the putative activation domain of eleven hevea *Pto*-RGCs. The Logo sequence consists of stacks of letters, one stack for each position in the sequence. The overall height of each stack indicates the sequence conservation at that position (measured in bits), whereas the height of symbols within the stack reflects the relative frequency of the corresponding amino acid at that position (Crooks et al, 2004). The autophosphorylation sites in the activation domain of *Pto* (Sessa et al, 2000) are indicated with *grey circles*.

the entire hevea *Pto*-RGCs presented a two amino acid deletion (subdomain V) and a three amino acid insertion (subdomain VIa) with regard to the Pto protein. We found that the two amino acid deletion was also present in *Pto*-RGCs from other monocot species such as *Solanum habrochaites* (GenBank accession No. AAK11567), *S. berthaultii* (GenBank accession No. AAK82689), *Phaseolus vulgaris* (GenBank accession No. AAK52079), *Oryza sativa* (GenBank accession No. XP476621) and *Tritium aestivum* (GenBank accession No. AAL51075). This deletion was also present in *Pto*-RGCs from other dicot species such as *Arabidopsis thaliana* (GenBank accession No. NP197789) and *Cucumis sativus* (GenBank accession No. AAP57674) but absent in *Phaseolus vulgaris* (GenBank accession No. AF363819). The extent and significance of this polymorphism in both monocot and dicot *Pto*-RGCs awaits further research. In the case of the three amino acid insertion, it is present in *Pto*-RGCs from other monocot and dicot species but absent in *Pto*-RGCs from the Solanaceae family (Vleeshouwers et al, 2001).

In order to highlight the *Pto* autophosphorylation sites that are conserved in the hevea *Pto*-RGCs and other critical residues for *Pto* function located in the activation domain, a sequence Logo was generated with the hevea *Pto*-RGC products and it is shown in Fig 2. Of the three *Pto* autophosphorylation sites (Thr195, Ser198 and Thr199) conserved in the hevea *Pto*-RGCs, Ser198 is required for the Avr*Pto*-*Pto*mediated hypersensitive response (Sessa et al, 2000) and it is present in the majority of hevea *Pto*-RGCs (Fig. 2).

The phylogenetic analysis of Fig. 3 shows that the seven hevea STK-like sequences identified as *Pto*-RGCs formed a cluster with the tomato Pto protein, which is supported by a high bootstrap value (99%). This result supported the designation of the seven hevea STK-like sequences as *Pto*-RGCs. Regarding the other hevea STK like sequences, four of them were related to receptor-like kinases as previously observed in the BLASTX results and the remaining sequence STK43 formed a highly supported (with bootstrap value 69%) cluster with a receptor-like kinase*a* (Fig. 3). This phylogenetic tree also showed that the protein kinase region used for its construction contains sufficient sequence information to represent clusters defined by analysis with full sequence data of the kinase catalytic domain (Hardie, 1999). Furthermore, phylogenetic analysis of the hevea *Pto*-RGCs with *Pto*-RGCs from different plant species (Fig. 4) revealed that the hevea *Pto*-RGCs were more closely related to *Pto*-RGCs from other plant species than each

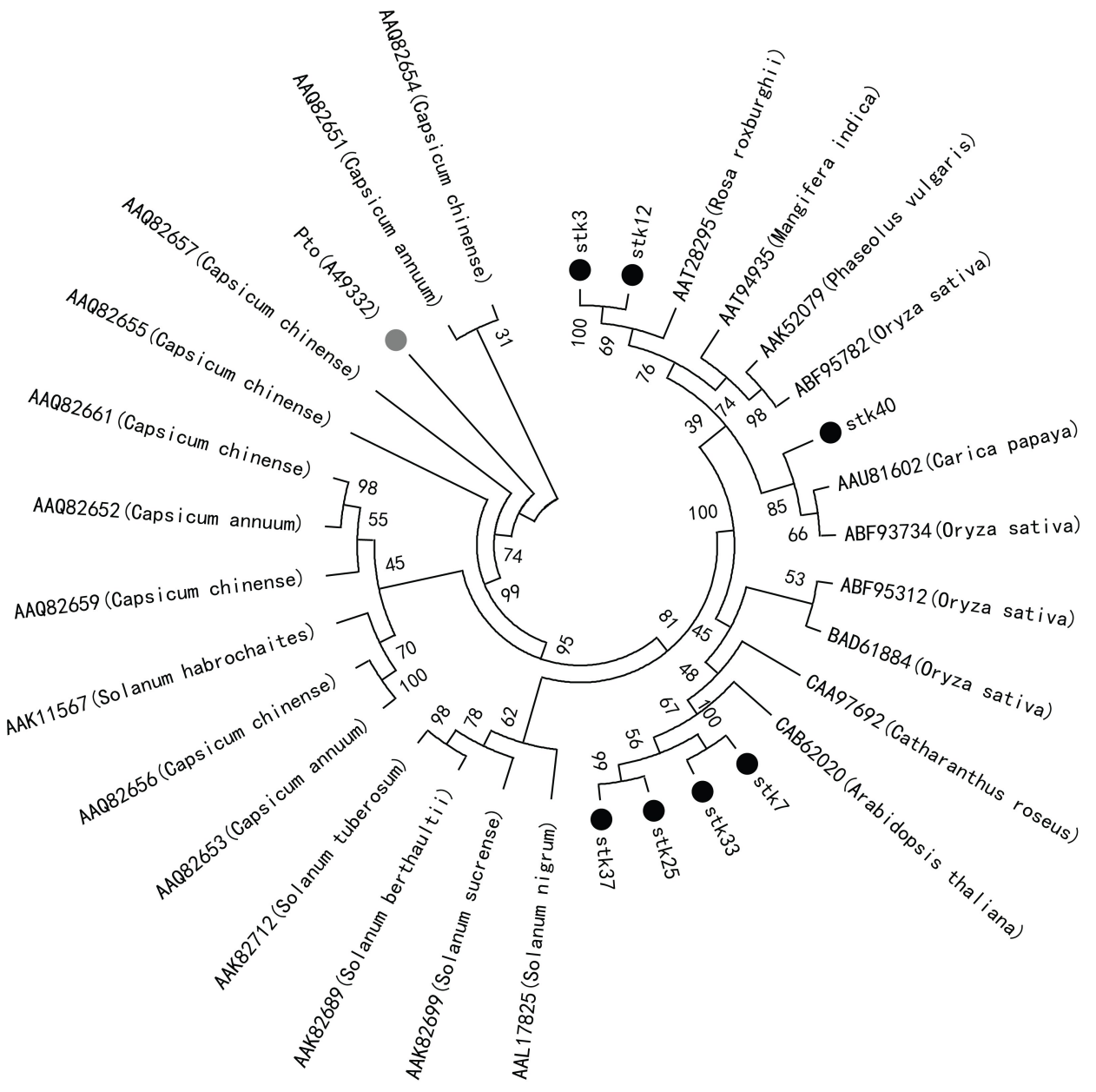


Fig. 3　Neighbor-joining phylogenetic tree based on the Clustal X alignment of serine/threonine kinases from *Arabidopsis thaliana* (Hardie, 1999; Hartwell et al, 1999), tomato Pto disease resistance protein, *Pto*-RGCs (black circles) and other STK-like sequences (grey circles) from hevea. Names of STKs are given for each sequence followed by GenBank accession numbers in parentheses. Amino acids from the end of subdomain I to the start of subdomain IX of STKs were used for the analysis. The numbers above the branches indicate the percentage of 1 000 bootstrap replications supporting the particular nodes.

other. Another interesting finding was that the clades where the hevea *Pto*-RGCs were grouped (Ⅰ, Ⅱ and Ⅲ) also contain *Pto*-RGCs from different species, suggesting that the origin of this type of sequence may have preceded the divergence of monocot and different plants.

## 3　Discussion

There is evidence that *Pto*-RGCs are highly conserved in many plant species. Southern hybridization

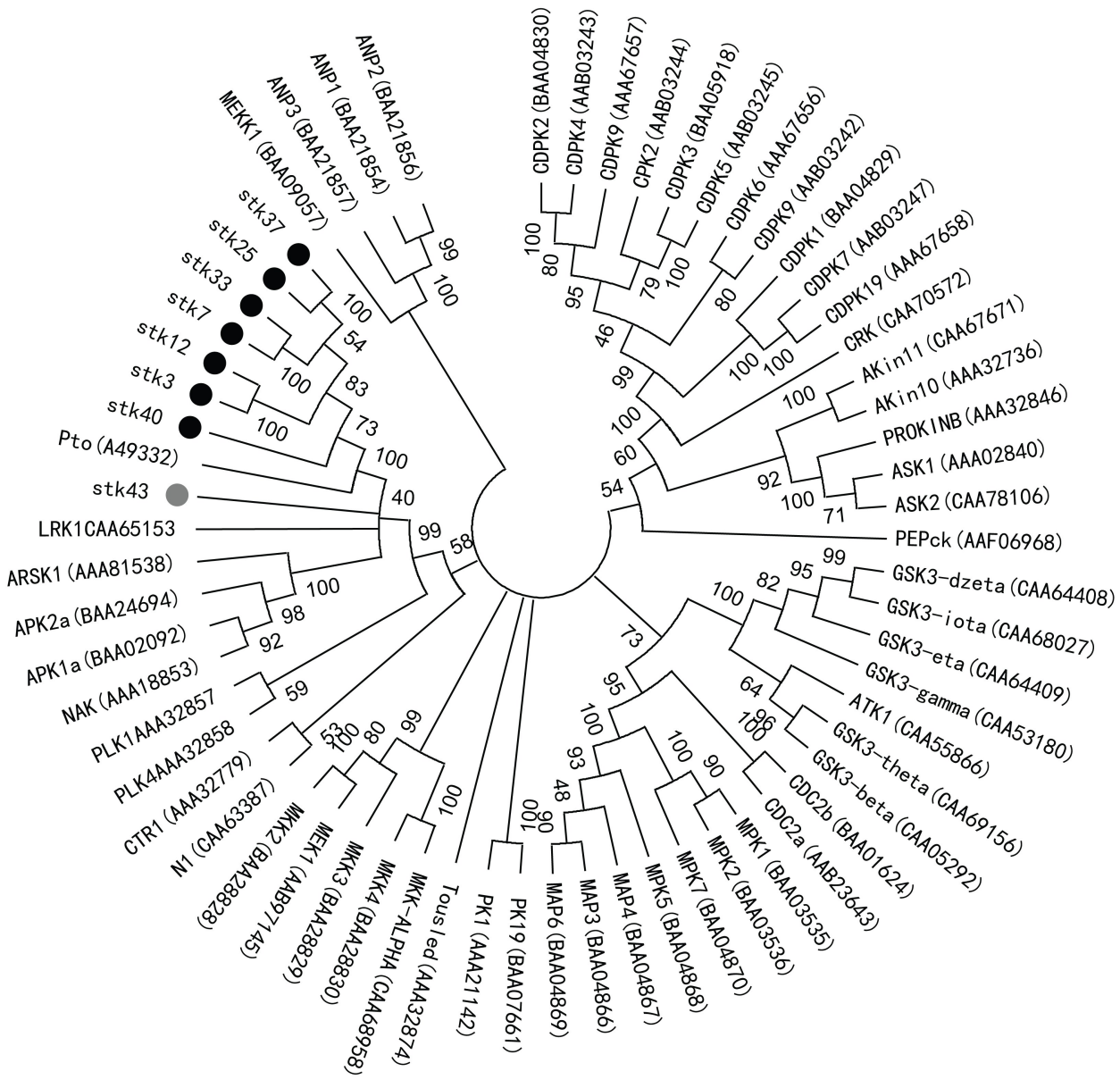


Fig. 4 Neighbor-joining phylogenetic tree based on the Clustal X alignment of tomato *Pto* disease resistance protein, *Pto*-RGCs from hevea (black) and other *Pto*-RGCs from monocot and dicot species. The name of the Pto protein is in bold and GenBank accession numbers are given for each sequence followed by species name in italics. Amino acids from the end of subdomain I to the start of subdomain IX of STKs were used for the analysis. The numbers above the branches indicate the percentage of 1 000 bootstrap replications supporting the particular nodes.

using the tomato *Pto* gene as probe revealed the presence of *Pto*-RGCs in many plant species such as *Arabidopsis*, bean, soybean, pea, rice, maize, barley, wheat and sugarcane (Martin et al, 1993). Recent studies report the cloning and characterization of *Pto*-RGCs from potato, bean and grapevine (Vleeshouwers et al, 2001; Vallad et al, 2001; DiGaspero & Cipriani, 2003). Furthermore, other *Pto*-RGC sequences from different plant families have been deposited in the GenBank (http:// www.ncbi.nlm.nih.gov), however these sequences have not been characterized. In this study, a set of *Pto*-RGC sequences and other STK-like sequences were identified from the hevea cultivar 'XJ000072'. These sequences were isolated by PCR using a pair of

degenerate primers previously designed and used by Vallad et al (2001) to isolate *Pto*-RGCs from bean. In total, seven distinct *Pto*-RGC sequences and five other STK-like sequences were identified in the hevea genome. These sequences were isolated by PCR using a pair of degenerate primers previously designed and used by Vallad et al (2001) reported the identification of a lower number of *Pto*-RGCs (five distinct sequences sharing from 56.9% to 63.9% amino acid identity with *Pto*) and no further cloning of other STK-like sequences. In this study, the PCR annealing temperature was lower (45 ℃) in comparison to the previous study (60 ℃), which may explain the broader diversity of STK-like sequences isolated in hevea. This low PCR annealing temperature could explain the isolation of the receptor-like sequence or the protein-like sequence, which was quite divergent from the rest of the hevea STK-like sequences isolated (*Pto*-RGCs and RLKs) (Fig. 3). Overall, our data demonstrate that the degenerate primers used are capable of amplifying *Pto*-RGCs and other types of STK-like sequences from a monocot species.

The complete genome sequence of *Arabidopsis* (genomesize of 130 Mbp) revealed the presence of 15 *Pto*-RGCs (Arabidopsis Genome Initiative 2000), while a draft of the rice genome sequence (genome size of 420 Mbp) revealed a similar number of *Pto*-RGCs with fourteen. These data indicate that the number of *Pto*-RGCs in these two plant genomes is conserved even though the rice genome is 290 Mbp larger than *Arabidopsis*, and also indicate that the number of *Pto*-RGCs in a plant genome is small in comparison to the NBS-LRR class of *R* genes, which has a large number of divergent genes in the *Arabidopsis* and rice genomes, with 149 and 480 genes, respectively (Meyers et al, 2003; Zhou et al, 2004). The genome size of hevea is estimated to be ~ 2 100 Mbp (Leitch et al, 1998), assuming that the number of *Pto*-RGCs in a plant genome do not increase significantly according to the genome size, then it is possible that in hevea the number of *Pto*-RGCs could be similar to *Arabidopsis* or rice. Hence, it is tempting to speculate that the number of *Pto*-RGC sequences identified in this study represents a significant proportion of the total number of *Pto*-RGC sequences in the hevea genome.

All hevea *Pto*-RGC products displayed conserved serine/threonine kinase sub-domains (Hanks & Quinn 1991), suggesting that the uncovered genes are likely to encode active kinases. Moreover, most residues of the Pto activation domain involved in pathogen recognition and HR induction (Pedley & Martin, 2003) are highly conserved in hevea *Pto*-RGCs suggesting that these residues might play a similar role in hevea. Indeed, the cloning of the full cDNA sequence and protein expression of these hevea *Pto*-RGCs will allow the possibility to answer some fundamental questions regarding for example, whether the *Pto*-RGCs encoded proteins are auto-phosphorylated *in vitro* and also whether substitution of tyrosine by aspartate in the corresponding site of *Pto* (Tyr207) will lead to a HR-like induction. Regarding the other STK-like sequences reported in this study, some of them are similar to receptor like kinases that are known to be involved in the response to pathogens, for example hevea STK8, STK15, STK17 and STK48 were related to the Constitutive triple response 1 (CTR1) group. CTR1, a member of this kinase group in *Arabidopsis*, also CTR1 is the immediate downstream target of ethylene receptors in *A. thaliana*, a putative Raf-like MAPK kinase kinase. Other interesting example is STK43, which was related to the leucine-rich repeat kinase1 (LRK1) group. LRK1 is an LRR-RLK isolated from *Arabidopsis thaliana*, and expression of the gene is induced by ABA, dehydration, high salt, and low temperature (Hong et al, 1997), but the function of RPK1 was still unclear. The role of other hevea STK-like sequences in disease resistance remains to be determined.

Phylogenetic analyses of *Pto* and *Pto*-RGC sequences have suggested that these sequences form a unique group of kinases in plants (Vallad et al, 2001; Vleeshouwers et al,2001). In agreement with this finding the hevea *Pto*-RGCs formed a highly supported group with the *Pto* disease resistance protein (Fig. 3) suggesting that these sequences share a common evolutionary origin with the tomato Pto protein and possibly a similar function in disease resistance. Furthermore, phylogenetic analysis of *Pto*-RGCs from different *Solanum* species has revealed that *Pto* orthologue genes are more

similar than paralogues suggesting that the origin of *Pto* could predate the radiation of *Solanum* species (Vleeshouwers et al, 2001). This ancient origin of *Pto* is further supported by the fact that both *Pto* and a *Pto* orthologue (*LhirPto*) are functional in *Nicotiana benthamiana* (Riely & Martin, 2001). Additional evidence of this ancient origin is the presence of *Pto*-RGCs in other dicot species and also monocots that have been recently deposited in the GenBank (http://www.ncbi.nlm. nih. gov). The phylogenetic analysis of Fig. 4 supports and extends these previous observations since all hevea *Pto*-RGCs were grouped in clades that contained *Pto*-RGCs from both monocot and dicot species suggesting that the origin of this type of sequence might have predated the divergence of monocot and dicot plants which took place about (200 ± 40) million years ago (Wolfe et al, 1989).

The tomato Pto protein is capable of recognizing at least two Avr proteins (Avr*Pto* and Avr*Pto*B) from *P. syringae*(Kim et al, 2002). Surprisingly, these two Avr proteins share limited sequence similarity. This dual recognition specificity has also been reported in R proteins of the NBS-LRR class, for example the Rpm1 protein from *Arabidopsis* confers resistance to *P. syringae* and recognizes two different avirulence proteins, AvrB and AvrRpm1 (Bisgrove et al, 1994). Another interesting example is the *Mi*-1 gene from tomato, which confers resistance to a nematode and an aphid pest (Vos et al, 1998). This dual (and perhaps even multiple) pathogen recognition specificity for a single R protein may prove to be common in *R* genes (Martin et al, 2003) and raises the possibility that *Pto* may confer resistance to pathogens other than bacteria. Whether the hevea *Pto*-RGCs are involved in conferring bacterial resistance as in tomato or are involved in conferring resistance to other types of pathogens will require functional analysis, which could be carried out with genetic complementation or loss of-function experiments. In the case of genetic complementation, the hevea *Pto*-RGCs could be used as probes to screen a hevea BIBAC library for the isolation of BIBAC clones containing *Pto*-RGCs. These *Pto*-RGC-BIBAC clones could be used to transform a hevea disease susceptible cultivar using *Agrobacterium tumefaciens* (Huang et al, 2010). These experiments would lead to a collection of *Pto*-RGC-BIBAC transgenic lines ready to be used for disease resistance tests. The BIBAC technology coupled with *Agrobacterium*- mediated transformation not only promises to unravel the function of hevea RGCs but also the development of disease resistance in this crop. In the case of the loss-of-function strategy, the hevea *Pto*-RGC sequences could be used in RNA interference (RNAi) constructs (Waterhouse & Helliwell, 2003) in order to silence their corresponding targets in a resistant genotype. Those resistant plants that show disease symptoms after the infection with a particular type of pathogen would allow the identification of an *R* gene. The RNAi technology has been recently used to determine the function of genes involved in disease resistance in barley (Douchkov et al, 2005). The hevea *Pto*-RGCs could also be used to produce molecular markers tightly linked to R-genes for genomic mapping and positional cloning. In this respect, several RGCs of the NBS-LRR class have been shown to be quite useful as molecular markers to assist the isolation of functional *R* genes through map-based positional cloning (McDowell et al, 1998; Zhao et al, 2005).

The *Pto* gene is considered as a promising candidate for engineering broad-spectrum pathogen resistance in tomato since plants over-expressing this gene display resistance to both bacterial and fungal pathogens (Tang et al, 1999). Moreover, expression of *Pto* mutants such as *pto**Thr*204*Asp* or *pto**Tyr*207*Asp* can constitutively activate a HR-like response in the absence of *P. syringae* (Rathjen et al, 1999). Expression of these engineered *Pto* genes under the control of a defined inducible promoter has been considered as another promising strategy to protect crops against pathogens through the hypersensitive response (Rathjen et al, 1999). The cloning of the full cDNA sequences of the hevea *Pto*-RGCs will permit assessing their potential to confer disease resistance using the strategies mentioned above.

In summary, this study has uncovered a set of hevea *Pto*-RGC sequences and provided the first insights about their amino acid sequence structure and evolution. The presence of several conserved amino acids in the

hevea *Pto*-RGCs that are crucial for *Pto* function, and the fact that these sequences were phylogenetically closely related to *Pto*, make of them a valuable sequence resource for plant-pathogen interaction studies in hevea. The hevea *Pto*-RGCs could be used to gene rate not only a collection of BIBAC clones or RNAi constructs for functional analysis but also they might be useful as molecular markers for genetic mapping. The availability of these sequences will facilitate the cloning of their corresponding full gene sequences, which in turn will allow further genetic and biochemical characterization that may lead to the development of specific or even broad-spectrum pathogen resistance in hevea. Moreover, the other hevea STK-like sequences identified in this study may be used as a research platform for further studies in this crop.

In addition to their potential use for genetic improvement, RGCs also provide opportunities and tools to answer some fundamental questions about disease resistance genes, such as structure, *R* gene organization, distribution and evolution (Michelmore & Meyers 1998; Meyers et al, 2003). The use of PCR with degenerate primers targeting the highly conserved subdomains of STK proteins has also proven to be an efficient method for isolating *Pto* resistance gene candidates (*Pto*-RGCs) in bean and grapevine (Vallad et al, 2001; DiGaspero & Cipriani, 2003), indicating that this approach could be used to retrieve this type of gene from other plant species.

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(上接第 540 页 Continue from page 540)

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