第30卷 第1期
2011年 2月

华 中 农 业 大 学 学 报
Journal of Huazhong Agricultural University

Vol. 30 No. 1
Feb. 2011,124～127

刺梨抗白粉病分子机制的研究*

徐 强

华中农业大学园艺林学学院,武汉 430070

刺梨(*Rosa roxburghii* Tratt)属蔷薇科蔷薇属植物,是我国特色的新兴水果之一,其果实肉脆、甜酸、具浓郁的特殊香味,被誉为水果中的Vc之王,其防癌、抑癌和抗衰老等保健作用在国内外引起很大关注。但在人工栽培过程中,刺梨白粉病(Uncinula necator)发生较普遍,主要危害幼叶、花蕾和幼果,在温暖湿润的地方或季节危害更为严重。白粉病是蔷薇科众多经济作物中最严重的病害之一,在刺梨这种较为野生的果树上探索抗白粉病分子机制将对一些缺乏抗性资源的蔷薇科果树和花卉作物的抗白粉病育种具有指导意义。

本研究选用抗、感白粉病刺梨为材料(与贵州大学合作),从病理学、细胞学、遗传学和基因组学等角度,开展刺梨抗白粉病分子机制的研究。主要结果如下:

1. 研究了刺梨白粉病年发生规律和白粉菌形态结构,寄主与白粉菌互作的细胞学特征,以及在白粉菌接种后刺梨防御相关蛋白的表达情况。研究内容包括分生孢子梗的荧光观察;分生孢子的长、宽测量;菌丝的直径与隔膜;子囊孢子的长、宽测量;子囊果的大小等。刺梨在受到白粉菌侵袭时,在菌丝周围发现H_2O_2的迅速累积,在被侵袭部位观察到胼胝质的积累。刺梨的几丁质酶和葡聚糖酶的表达水平在白粉菌接种前后差异显著。

刺梨白粉菌生物学特性及其形态结构特征的鉴定为该菌正确分类提供了依据。

2. 克隆了126个抗病基因类似物(resistance gene analog,RGA),发现*RGA*基因在刺梨基因组成簇存在,具有快速重组与进化、减数分裂不稳定和进化模式高度复杂等特点,这些特点的形成与正向选择压力、平衡选择压力、重复序列的重组、点突变、以及转座子元件插入等分子事件有关。从刺梨基因组中,获得96个具有开放读码框架的*RGA*基因,其中34个*RGA*来源于抗病亲本,30个来源于感病亲本,32个来源于它们的F_1后代。通过比较发现抗病亲本和感病亲本序列之间的核苷酸同源性平均值是54%,稍高于抗病亲本的34个序列内部的同源性52%。系统进化分析可以把这96个基因明显分为两大类:一类与nonTIR类型*R*基因同源性较高且含有该类型*R*基因的特异结构域,而另一类则含有TIR类型*R*基因的特异结构域。遗传作图把这96个基因定位到3个连锁群:最大的含有23个标记,命名为CR1,基因主要来源于抗病亲本和F_1代;第2个连锁群CR2含有12个标记,全部来源于感病亲本;第3个连锁群CR3有6个标记。对这3个连锁群中的*RGA*基因的位置和进化树的位置进行比较分析发现:同一个进化枝上的*RGA*基因在遗传图上往往也紧密"捆绑"在一起,这是由于基因的串联重复(tandem duplication)之后序列分化造成的;另一种情况是来自明显不同进化枝的*RGA*基因在作图时定位在一块,形成杂合性基因簇(heterogeneous cluster),可能起源于*RGA*基因簇区域之间的异位重组(ecotopic recombination)。从亲本到F_1代,还观察到了*RGA*基因片段缺失的现象,与*RGA*基因的减数分裂不稳定有关。利用TAIL-PCR策略,在*RGA*基因侧翼分离到了1个转座子类似序列,Southern杂交分析发现该基因的拷贝数在抗病亲本中较高,在种内、种间拷贝数有很大的差异,转座子极有可能参与了抗病新位点的形成。利用已经发表的蔷薇科*RGA*基因,分析了来自刺梨、苹果、桃、梨、草莓、杏和李等228个*RGA*基因,在属间、种间进行了比较分析,探索了*RGA*存在共线性的可能。进化分析还发现位于第125的组氨酸处于正向选择,补充了产生抗病新位点的动力。

在植物上首次运用了重叠延伸法目的性克隆*RGA*基因,该方法可以避免兼并引物PCR的偏好

收稿日期:2010-12-03
* 2010年度全国百篇优秀博士学位论文(指导老师:邓秀新)
徐 强,博士,副教授. 研究方向:果树遗传育种. E-mail:xuqiang@mail.hzau.edu.cn

性。发现的 *RGA* 基因具有减数分裂不稳定性是国际上的首例报道。

3. 研究了植物免疫系统下游相关基因包括 *PTO*-like 蛋白激酶基因和防卫相关基因(defense-related genes)，这些基因以家族的形式存在于基因组，各成员之间多态性多为单核苷酸(SNP)多态性，发现 1 个基因响应于白粉菌侵袭。从刺梨抗病品种克隆了 30 个防卫相关基因，9 个 *PTO*-like 激酶基因，21 个病程相关蛋白基因(pathogenesis-related genes)，其中 12 个 *PR*2 基因，9 个 *PR*5 基因。多态性主要由单核苷酸位点(SNP)构成，包括点突变、小插入/缺失(InDel)构成。*PR*5 基因平均出现 1 个 SNP 频率为 59 bp，*PR*2 基因是 64 bp。基于 SNPs，进一步开发了 SNAP 标记，共设计了 23 对引物，最后 17 个标记可以在 F_1 群体定位。反向 Northern 表达分析表明，*PR*2 基因在接种后基本上不表达，*PR*5 基因中的 1 个在接种后的表达明显增强。

研究发现刺梨基因组中免疫相关基因从上游到下游即 *R* 基因→*STK* 基因→*PR*2 基因处于一个共进化体系。

4. 克隆了刺梨显著应答白粉菌侵袭的基因，并进行了验证。首先构建了富集抗白粉病相关基因的抑制消减(SSH)文库，反向 Northern 筛选后挑取表达量差异显著的克隆测序之后，发现一些是已报道抗病相关的基因，如 *PR*10、*P*450、泛素、*STK*-like kinase 基因、*Cf*-9 类似基因、*LRR* 受体类似激酶等，转录相关基因如 *NAC* 基因、Histone 组蛋白基因，甚至是转座子基因。但最为显著的一类是与光呼吸相关的基因如核酮糖-1，5-二磷酸羧化/加氧酶(Rubisco)、Rubisco 激活酶、乙醛酸转氨酶、谷氨酸转氨酶、甘油醛脱氢酶、铁氧还原蛋白和转运蛋白等。Real-Time PCR 验证和酶活性分析表明 3 个光呼吸重要基因 Rubisco、Rubisco 激活酶、乙醛酸转氨酶基因的表达明显响应于白粉菌接种，其中乙醛酸转氨酶基因在白粉菌接种处理中的表达量是对照的 40 倍，酶活性检测表明该酶在处理中的活性是对照的 25 倍。遗传作图发现这 3 个基因在刺梨基因组中聚类在一起。用 RACE 技术获得了这 3 个基因的全长，分别为 812 、935 和 1 163 bp。根据全长序列设计引物扩增这 3 个基因的 DNA 区域，比较它们在刺梨抗病品种贵农 6 号，感病品种贵农 5 号和贵农 1 号，白粉病免疫品种无籽刺梨，蔷薇科其他作物如苹果、桃、梨、月季基因组中的多态性，结果表明，抗病品种贵农 6 号和对白粉菌免疫的无籽刺梨在 Rubisco 基因位点的序列完全一样。

5. 提出了植物抗白粉病的一种新途径——光呼吸。光呼吸酶基因不仅对白粉菌侵袭有明显的应答反应，其酶的活性也显著升高；研究还发现这些光呼吸酶基因的转录显著受抗病信号分子水杨酸的诱导，且在刺梨抗白粉病过程中水杨酸和另一类抗病信号分子 H_2O_2 有积累现象。刺梨光呼吸途径在抗白粉病过程中扮演重要角色，是植物抗白粉病的一种新途径，因此本研究提出刺梨抗白粉病的分子机制——光呼吸途径。目前正在利用拟南芥野生型和抗病信号途径突变体开展刺梨光呼吸酶基因介导白粉病抗性的机理研究。

关键词　刺梨；白粉病；防卫相关基因；光呼吸；抗病基因类似物

Molecular mechanism of powdery mildew resistance in chestnut rose(*Rosa roxburghii* Tratt)

XU Qiang

College of Horticulture and Forestry, Huazhong Agricultural University, Wuhan 430070, *China*

Chestnut rose(*Rosa roxburghii* Tratt), belonging to *Rosa* genus of Rosaceae family, is a new promising fruit crop in China due to its fruits having high content of vitamin C and displaying high levels of superoxide disomutase (SOD) activity, which can delay senescence and prevent cancer. However, powdery mildew disease is common in the production area, especially when large areas of chestnut rose are cultivated. Damage caused by powdery mildews can be stunting and distort leaves, buds, growing tips, and fruit, the symptoms become more serious in areas with high humidity and hot weather. Powdery mil-

dew resistance breeding becomes one of the most important goals for various economical crops within *Rosaceae* family; understanding the molecular mechanism underlying powdery mildew resistance in a relative wild crop would be beneficial to the molecular breeding of disease resistant cultivars of fruit and ornamental crops in Rosaceae family.

Using the powdery mildew resistant and susceptible genotypes as materials, phytopathological, cytological, genetics, and genomics researches were carried out to understand the mechanism involved in powdery mildew resistance in chestnut rose. The main results are as following:

1. The annual life-cycle of powdery mildew fungi and the shapes of fungi in different stages, the host-microbe interaction, and the expression of defense-related enzymes upon powdery mildew infection were studied. The experiments included the observation or determination of conidiophores, conidia, ascospore, hyphae and ascocarps. Moreover, H_2O_2 were observed accumulating near around the hyphae attacking sites, and the callose was detected by fluorescence analysis. Chitinase and glucanase were significantly responded to the innoculation of powdery mildew.

The biological habits and morphological characterization of powdery mildew fungi in chestnut rose studied herein provided evidence for the correct classification of this fungus.

2. One hundred and twenty six resistance gene analogs (RGAs) were cloned from chestnut rose genome. The *RGA* genes, clustered in the genome, are rapidly evolved, meiotically instable, and evolutionarily complex. The reasons for these characteristics and the generation of new resistance specificity could be the positive selection, balancing selection, recombination, point mutation, and even transposable elements. From chestnut rose, 96 resistance gene aanalogues (RGAs) were cloned and characterized, of which 34 were derived from resistant parent, 30 from susceptible parent, 32 from F_1 progeny. Comparison revealed that the nucleotide similarity between the resistant and susceptible parent is averaged at 54%, higher than that within resistant parent. Phylogenetic analysis divided these 96 genes into two groups; one group showed high homology with non-TIR resistance genes, the other is highly homologous to TIR resistance gene. Genetic mapping divided these 96 genes into 3 linkage groups: the biggest group contained 23 genes, designated as CR1; the second one contained 12 genes, all were from susceptible parent; the last group CR3 contained 6 genes. Comparing the positions of genetic map with phylogenetic tree, the *RGA* gene from a phylogenetic clade tended to cluster in genetic map, which was caused by tandem duplication and diversification. The other case is *RGA* genes from different clades clustered together in the genetic map and formed heterogeneous cluster, this might caused by ecotopic recombination. From the parents to F_1 progeny, deletion of *RGA* gene's fragment was observed, which may related with meiotic instability. Using TAIL-PCR strategy, a retrotransposon-like gene flanking *RGA* gene was isolated, Southern analysis revealed that the copy number of retrotransposon-like gene is relative large with great difference among different materials. Based on the published Rosaceae *RGA* genes from chestnut rose, apple, peach, pear, strawberry, apricot and plum, were comparatively analyzed on genus and species level. The synteny of *RGA* gene between different genus were discussed, and the 125 H site was identified to be under positive selection by evolutionary prediction analysis.

This is the first report of using overlap extension strategy for target isolation of *RGA* genes in plants; this method can overcome the problem of bias amplification of degenerate PCR. Also this is the first report of meiotic instability for *RGA* gene.

3. The downstream components of plant immunity system in chestnut rose, including the *PTO*-like protein kinase and defense-related genes, were investigated. The immunity related genes mostly exist in the genome as gene family. Among the members, single nucleotide polymorphisms (SNPs) are more

prevalent than other sequence polymorphisms. Several members are obviously responded to the powdery mildew attack. From the resistant cultivar, 30 defense-related genes (*DR* genes) were cloned, including 9 *PTO*-like kinase genes, 21 pathogenesis-related genes (12 *PR*2 genes and 9 *PR*5 genes). The polymorphism of gene family was mainly composed of single nucleotide polymorphisms (SNPs). The average frequency for *PR*5 gene was one SNP per 59 bp, 64 bp for *PR*2 genes. Based on the SNPs, SNAP markers were developed, 23 primer pairs were designed and 17 markers were finally mapped in F_1 population. Reverse Northern revealed that all *PR*2 genes were not induced significantly after innoculation, while one *PR*5 gene's expression was significantly enhanced.

The immunity related genes in chestnut rose genome from upstream to downstream, i. e. *R* gene→*STK* gene→*PR*2 gene, were predicted to be involved in a co-evolution system.

4. Highly expressed genes activated by powdery mildew pathogen attack were cloned and characterized. Suppression subtraction hybridization (SSH) library which enriched powdery mildew responded genes was constructed, and reverse Northern technology was used to screen the clones from the library. Sequencing the differentially expressed clones revealed many genes highly homologous to resistance-related genes reported previously, such as *PR*10、*P*450、*STK*-like kinase gene, *Cf*-9-like, *LRR* receptor-like genes, transcription factor *NAC* gene, and even the transposon elements. The most noticeable genes appeared in the library is photorespiratory-related genes, such as ribulose bisphosphate carboxylase (Rubisco), Rubisco activase, glyoxylate aminotransferase, glutamine synthetase, glyceraldehyde dehydrogenase, ferredoxin, transport protein, etc. Real-Time PCR were used to verify the three most important photorespiratory genes, which revealed that expression levels of Rubisco activase, glyoxylate aminotransferase were significantly enhanced after innoculation. The enzymatic activities of these three photorespiratory genes were also verified to be enhanced significantly after innoculation by spectrophotometric analysis. For example, the transcription level of aminotransferase gene in powdery mildew infected material was as high as 40 folds of that in control material, and the enzymatic activity of aminotransferase in treated material was 25 folds higher than that in control material. Genetic mapping showed that the three photorespiratory genes exist in the chesnut rose genome as gene cluster. The full length cDNA of these three genes were further obtained by RACE strategy. Moreover, the DNA regions of these genes among the genomes of chestnut rose with different genotypes and rosaceae fruit crops were compared. Interestingly, the sequence of ChrRBCs in Guinong No. 6, which is highly resistant to powdery mildew, is identical with that in Wuzi Cili, a genotype immune to powdery mildew.

5. A new mechanism for powdery mildew disease resistance in plants, i. e. photorespiration, was proposed. Photorespiratory genes are not only highly responded to powdery mildew pathogen attack with the transcription and the enzyme activites highly induced; but also, we found that the transcriptions of photorespiratory genes are significantly induced by resistance signal salycilic acid, and the salycilic acid with another signal peroxide was accumulated significantly in the powdery mildew infected samples. Altogether, photorespiratory gene may be a kind of new resistance gene; therefore photorespiration maybe a new mechanism for plant to defense against pathogen attack were proposed. We are now carring out a further research on *Arabidopsis* to verify the new function of photorespiratory genes from chestnut rose, and to investigate the molecular mechanism of photorespiratory gene functioned in powdery mildew resistance.

Key words　*Rosa roxburghii*; powdery mildew; defense-related genes; photo-respiratory; resistance gene analogues

（责任编辑：杨锦莲）