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Chestnut Rose (*Rosa roxburghii* Tratt): a Promising Genetic Resource for Fruit and Ornament Exploitation in China

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ABSTRACT

Chestnut rose (*Rosa roxburghii* Tratt) brightens the spring garden with its attractive flowers. Moreover, it shows promising prospects in fruit exploitation due to its high content of vitamin C (2054-3541 mg/100g FW), very high superoxide dismutase activity, attractively senescence-retarding and cancer-preventing effects. Considerable efforts have focused on its research during the past two decades. To satisfy the increasing demand of exploitation of this plant, this overview serves chiefly to demonstrate: 1) the nutritional and medicinal values of chestnut rose fruit, as well as its ornamental features; 2) propagation method; 3) the genetic diversity of germplasms and its evaluation using morphological traits and molecular markers; 4) inheritance tendency of some agronomical traits; 5) immature embryo *in vitro* culture technique, high efficient micropropagation and assessment of the genetic stability of cultures; 6) accumulating process and characteristics of vitamin C during fruit development, and molecular cloning and characterization of L-galactono-1,4-lactone dehydrogenase (GalLDH), a key enzyme catalyzing the terminal step of vitamin C biosynthesis; and 7) screening of molecular markers linked to resistance to rose powdery mildew, and cloning and analysis of resistance gene analogs in chestnut rose.

Keywords: genetic diversity, *in vitro* culture, linked marker, molecular cloning, propagation, resistance gene analogs (RGAs), vitamin C
Abbreviations: AFLP, amplified fragment length polymorphism; **BSA**, bulked segregant analysis; **CAPS**, cleaved amplified polymerphic sequence; **GalLDH**, L-galactono-1,4-lactone dehydrogenase; **MAS**, marker-assisted selection; ***PR***, pathogenesis-related; **QTL**, quantitative trait locus; ***R***, resistance; **RACE**, rapid amplification of cDNA end; **RAPD**, randomly amplified polymorphic DNA; **RGAs**, resistance gene analogs; **RFLP**, restriction fragment length polymorphism; **SNAP**, single nucleotide-amplified polymorphism; **SSH**, subtractive suppression hybridization; **STS**, sequence tagged site; **TIR**, toll and interleukin-1 receptor

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INTRODUCTION

The genus *Rosa* has about 200 species, many of which are of excellent ornamental importance. Chestnut rose (*Rosa roxburghii* Tratt) is native to China and is widely distributed in the south-west, central-south, south and north-west provinces of this country. In the 1800's William Roxburgh, an assistant surgeon to the East India Company came across this rose in a garden of Canton, China, where it had been grown for generations as an ornamental plant. He sent it to the Calcutta Botanic Garden, India. From there it reached England in 1820 and quickly traveled on to America for ornamental use. To celebrate William Roxburgh, this rose was named *Rosa roxburghii* Tratt. Interestingly, it was also labeled as chestnut rose in 1904 because of the bristly spikes covering the flower buds and fruits, a trait completely unique in the rose family (Fangan 1988).

Chestnut rose grows into a large shrub with small light green attractive foliage, and carnation-like roses in spring. It brightens the spring garden with its colorful, prolific, multi-corolla flowers (Xiang *et al*. 1987; Ji and Li 1998). Its fruit contains very high levels of vitamins and minerals (Luo 1984; Zhu *et al.* 1984). Furthermore, it was proved to be a food that is capable of reducing the incidence of cancer and also as a means of halting or reversing the growth of cancers (Wu *et al.* 1986a, 1986b; Ma *et al.* 1997). The seed is a good source of vitamin E, and it can be ground into a powder and mixed with flour or added to other foods as a supplement (Facciola 1990). It was documented that this plant is free from rose blackspot disease, caused by the fungus *Diplocarpon rosae* (Wiggers *et al.* 1997).

Due to that mentioned above, chestnut rose shows a great potential for the exploitation of its fruits and for ornamental planting, and has brought about statewide interest. Intensive efforts were focused on its exploitation in the past two decades (Shi *et al.* 1991; Fan *et al.* 1997; Wen *et al.* 2004). Also, it has been considerably documented in germplasm and biotechnology during the past several years

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(Wen *et al.* 2004; An *et al.* 2005a, 2005b; Wen *et al.* 2005; Xu *et al.* 2005, 2006a, 2006b, 2007). To meet the increasing demand of exploitation of this rose, the present work provides an introduction to this plant, and overviews the present scientific progress of its biotechnology.

CHESTNUT ROSE – THE PLANT

This rose is unique in many ways. The canes of chestnut rose are a tawny brown, turning grayish with age. The full, dense, mounding bush grows to approximately four to five feet tall and three to five feet in canopy diameter. The odd, pale brown bark of the branches, combined with foliage divide into many small leaflets, and makes this rose a fascinating plant, especially if allowed to reach its mature size. It may take a few years to reach its full size, but it makes a great carefree addition to a garden. A feature, unusual in species roses, is that the large initial spring bloom is followed by sporadic repeat blooms until autumn. The red, pink, white, and lightly fragrant flowers open irregularly from mossy-looking buds throughout the growing season, and are followed by bristly hips that resemble a chestnut. The two to three inch blooms are considerably fragrant, and very attractive to bees and butterflies. Besides uni-corolla flowers, some genotype can bear multi-corolla ones (Ji and Li 1998). If planted in the shade, it will be much smaller than if it is planted in full sun.

The fruits have diverse shapes: globular, spindly, oblate or columniform (Wen *et al.* 2003, 2004). There is a layer of flesh surrounding many seeds, and is thus also named as hip. Some care has to be taken when eating this fruit, because there are many thorns on the peel. The mature fruits smell like ripe pineapples, are aromatic and have a sour-sweet taste. They become yellow or light yellow when ripe. The fruit is characterized by a very rich source of vitamins and minerals, especially vitamins C and P, flavanoids and other bio-active compounds (Luo 1984; Luo *et al.* 1984). The vitamin C content of chestnut rose fruits are 2054-3541 mg/100g FW (Zhu *et al.* 1984), and the vitamin P titer is as high as 12,895 mg/100g FW, which is about 120 times higher than that of citrus fruit (Luo 1984). Also, the fruit is a good source of essential fatty acids, which is fairly unusual for a fruit. The total sugar contents are 8.96%-17.05%, among which fructose, glucose and sucrose are 52.3%, 39.7% and 8.0%, respectively (Guo 1986). It is proved that the fruit juice is capable of reducing the incidence of mice cancer and also as a means of halting or reversing the growth of cancers (Wu *et al.* 1986a, 1986b; Shi *et al.* 1991; Ma *et al.* 1997), may enhance immunity and can be used to combat stress and aging (Burke *et al.* 2005). Toxicity of Pb to mice can be remarkably alleviated when the juice is used as feed (Wu *et al.* 1986a). Additionally, considerable high superoxide dismutase (SOD) activity (ca. 54,000U/100g FW) can be detected in this fruit (Wu 1986).

PROPAGATION

Chestnut rose shows for a considerably short juvenile span, therefore both vegetative and seed propagation can be used to multiple plants. Usually, the latter is essential for hybriddization breeding. Seed often takes two years to germinate, which may be attributed to the immature embryo and hard seedcoat. One possible way to shorten this time is to stratify at 4°C for 4-6 weeks and then place it in damp peat or soil at a temperature of 23-27°C, after which seeds can germinate in 2-3 weeks. Plant out in spring if the plants are large enough to handle.

Also, it can be vegetatively propagated by cutting, layering or grafting. Cuttings of half-ripe wood or mature wood show the highest percentage of success. Pencil-thick shoots that are about 20-25 cm long should be selected in early autumn and planted in a sheltered position at 25±2°C (Xiang 1984). For graft propagation, the best stock is *R. multiflora* Thumb var. *cathayensis*, and the highest success rate can be obtained from it. Furthermore, the most vigorous plant and prolific fruits can be obtained from this stock (Liu 1984).

GENETIC GERMPLASM AND BIO-DIVERSITY

Arduous efforts to investigate and collect wild germplasm based on visible characteristics, especially on agronomic traits of fruits started in the early 1980s in China (Zhu *et al.* 1984), and a total of five cultivars, three taxa and some elite genotypes were obtained (Xiang *et al.* 1987; Fan *et al.* 1997; Ji and Li 1998). Detection of genetic differences and elucidation of genetic relationships between genotypes are the prerequisites for full utilization of this genetic resource and protection of its proprietary right. Some morphological traits have been employed to describe genotypes (Xiang *et al.* 1987; Ji and Li 1998). Previously, chestnut rose breeding programs were only based on plant growth vigor and fruit characteristics. Wen *et al.* (2004) demonstrated that these genotypes could be reliably discriminated by RAPD or AFLP markers. The polymorphic pattern derived from AFLP can be employed as a powerful means to solve the ambiguities encountered by commercial growers. Genetic variation of 30 wild populations from mainly distributed regions in China was investigated by RAPD markers. The highest variations are among the wild accessions from Guizhou Province, and accessions from west Hubei Province also showed considerably great polymorphisms (Wen *et al.* 2003).

R. roxburghii Tratt f. *normalis*, from which all comercial cultivars were derived in China, is quite different from multiple-corolla chestnut rose (*R. roxburghii* Tratt f. *roxburghii*) and white-flower chestnut rose (*R. roxburghii* Tratt f. *candida*) in floral characteristics. Multiple-corolla type is characterized by bearing replicated-petal and red flowers, thus this taxon shows a great potential for ornamental plant breeding (Zhu *et al.* 1984; Ji and Li 1998; Wen *et al.* 2006). It was first named as *R. roxburghii* Tratt f. *roxburghii* because William Roxburgh imported this type to Europe (Matthews 1994). Once, it had always been assumed to be the proto-species, whereas the uni-corolla type was regarded as the variant deriving from it, and the latter was named *R. roxburghii* Tratt f. *normalis* (Yu 1985; Matthews 1994). However, the highest percentage of polymorphic band (PPB) value in RAPD markers (Wen and Deng 2003a) and a considerably high PPB value in AFLP markers among the genotypes of *R. roxburghii* do not support the assumption. When the morphological features are taken together, Wen *et al.* (2004) deduced that *R. roxburghii* Tratt f. *normalis* should be the prototype, and multiple-corolla and white-flower types would be the variants, which coincides with a study on morphology (Ji and Li 1998).

Seedless chestnut rose characteristically yields a seedless chestnut-like hip, which is the only taxon showing parthenocarpic ability at present among chestnut rose and its relatives (Zhu *et al.* 1984; Ji and Li 1998). It has been conferred high importance for genetic improvement, although its fruit shows a fairly lower vitamin C content (Zhu *et al.* 1984). It was catagorized as chestnut rose in practical production for its hip characteristics, and named *R. sterilis* (Ji and Li 1998). Surprisingly, the closest similarity between this taxon and *R. kweichonensis* was observed in both RAPD and AFLP analysis (Wen *et al.* 2004), which is in agreement with its morphological description (Yu 1985; Ji and Li 1998). Some scientists presumably regarded it as the natural hybrid between *R. roxburghii* and its relative of *Rosa* (Ji and Li 1998). Based on morphological, cytological and molecular investigations, Wen *et al.* (2004) regarded it as a male sterile mutant of *R. kweichonensis*.

INHERITANCE TENDENCY OF SOME AGRONOMICAL TRAITS

Inheritance of some agronomical traits, e.g. fruit size, concentration of vitamin C, tannins, soluble solids and organic acids, etc, have been investigated. Using selected wild geno-

types as parents, the first filial (F_1) progenies were employed to investigate their inheritance tendency. The scored values of all these traits are continuous within the population, and governed by polygenes, however, environmental factors also demonstrated considerably high effects on the phenotypic values. Among these parameters, vitamin C content and fruit size showed a certain transgressive inheritance. Compared with the mean value of parents, most of the F_1 progenies yielded bigger fruits with higher vitamin C content. Conversely, the mean concentration of tannin was lower than that of the average value of parents (Gao *et al.* 1986). Taken together, it is proposed that agronomical traits can be considerably improved by hybridization between selected wild lines. Unfortunately, the inheritance of ornamental traits has not yet been unraveled, and is an ongoing topic of research. Gao and Luo (1994) also preliminarily investigated the hereditary mode of fruit burrs. Chestnut rose burr is a dominant trait, and governed by two alleles, while the burr-free trait is recessive. However, more detailed investigations should focus on the genetic law of important agronomical and ornamental traits.

Chestnut rose is seriously disease-stricken by powdery mildew (*Sphaerotheca pannosa*) disease in cultivation (Pang *et al.* 1986; Xu *et al.* 2005; Wen *et al.* 2006). A total of 95 F_1 progenies of Guinong No. 6 (resistant) × Guinong No. 5 (susceptible), and 63 seedlings of their reciprocal cross were used to investigate the inheritance tendency of resistance to this disease. Based on the disease index, the resistance appears to be co-dominated by two types of genetic models: 1) two alleles which show an additive effect between them; and 2) multiple genes which may contribute to the continued disease index values within the same rating. Heredity of F_1 progeny disease index was significantly higher than the mid-parent value, which reveals an additive genetic effect as well as a non-additive genetic effect may confer resistance to this disease (Wen *et al.* 2005).

TISSUE CULTURE

In vitro multiplication can help in germplasm conservation and production of a large number of disease-free, true-to-type plants. Chestnut rose has been the subject of fewer *in vitr*o studies than other species of *Rosa*. Gao and Gao (1994) firstly reported a protocol for *in vitro* propagation using apical meristem tips. A micropropagation system based on axillary bud proliferation was also established and optimized for mature plants (Yan *et al*. 1996; Wen and Deng 2005a; reviewed in Khosh-Khui and Teixeira da Silva 2006). Unfortunately, explants or *in vitro* shoots showed serious browning due to a large quantity of phenolic compounds, and followed by shoot withering and necrosis. With the addition of 300-400 $mg.l^{-1}$ active charcoal in the media during explant inoculation and the following several multiplications, the detrimental effect may be significantly alleviated. For micropropagation, a suitable concentration of 6-benzyladenine added to the medium plays an important role in circumventing browning and improving multiplication.

Among the documented *in vitro* studies of *Rosa*, reports of regeneration from immature zygotic embryos are very limited (Rout *et al.* 1999). An efficient method for plantlet regeneration through organogenesis from immature embryos of chestnut rose had been developed using 35-55 days-old immature cotyledonary embryos of Guinong No. 5 and white-flower chestnut rose (Wen and Deng 2005b). This protocol may be employed to carry out embryo rescue in hybridization.

Genetic fidelity is of great importance in both micropropagation and germplasm conservation of plants. Callus is more prone to generate genetic variation. Cytological observation, RAPD and AFLP markers were employed to evaluate the genetic stability of Guinong No. 5 and white-flower chestnut rose callus induced from immature embryos (Wen and Deng 2003b). Aneuploids were commonly observed in callus of the 1st and 2nd cycle (9.43%), and in callus subcultured for 20 cycles, the varied cells counted for 20.75%, of which 15.09% were hypoploids. Using RAPD and AFLP marker, aberrant markers were scored in the two genotypes. To maintain genetic stability, the callus should not be subcultured for more than 10 cycles. The *in vitro* shoots that derived directly from adventitious buds around the well-organized axillary buds were also assayed using RAPD and AFLP markers, and no genetic variation at DNA sites was detected when the shoots were multiplied for as many as 25 cycles. Interestingly, as mentioned above, polymorphic RAPD and AFLP markers were detected in the callus (Wen and Deng 2003), which may reveal that the *in vitro* cultures of chestnut rose are not always genetically stable. To minimize the genetic variations, therefore, it is suggested that axillary buds should be used for multiplication and conservation.

VITAMIN C BIOSYNTHESIS AND ACCUMULATION

L-Galactono-1,4-lactone dehydrogenase (EC 1.3.2.3, GalLDH) is a key enzyme catalyzing the terminal step of vitamin C biosynthesis in higher plants (Pateraki *et al.* 2004). Using RT-PCR and rapid amplification of cDNA end (RACE) techniques, full-length cDNA of this enzyme was isolated from chestnut rose (GeneBank accession number: AY643403). Its expression patterns correlated with fruit vitamin C accumulation rates rather than the onset of ripening (An *et al.* 2004). Vitamin C is able to be synthesized effectively in fruits through the L-galactose pathway and the uronic acid conversion pathway, but mainly via the former (An *et al.* 2005a). Meanwhile, the vitamin C pool size was mainly determined by GalLDH activity.

The highest level of accumulated vitamin C was caused by three mechanisms in fruit (An *et al.* 2005b). High-level expression and long-term activity of GalLDH are the chief reasons for the persistent biosynthesis of vitamin C during fruit development. Also, both pathways working in the fruits may considerably contribute to a huge amount of vitamin C biosynthesis. In addition, transitory and low activities of ascorbic acid oxidase (AAO) and ascorbate peroxidase (APX) in the fruit suggest slow vitamin C degradation during fruit development.

MOLECULAR MARKERS LINKED TO RESISTANCE TO ROSE POWDERY MILDEW

Previous genetic analysis revealed that resistance was assumed to be conferred by two alleles (Wen *et al.* 2005). Guinong No. 6, an indigenous cultivar, demonstrated high resistance to rose powdery mildew disease. Utilizing a cross approach, the resistance of Guinong No. 6 may be introduced into Guinong No. 5, a susceptible and widely cultivated cultivar. Based on the double pseudo-backcross strategy, the derived F_1 population offers the possibility to unravel the genetic basis of resistance to this disease.

By a candidate gene approach with the aim to clone Resistance (*R*) genes, a total of 11 TIR-type and 23 non-TIR-type resistance gene analogs (RGAs) were cloned and characterized from chestnut rose resistant genotype using two PCR-based strategies including direct PCR and overlap extension amplification (Xu *et al.* 2005). The identity percentage between their deduced amino acids varied from 21% to 99%. Bulked segregant analysis (BSA) method was employed to screen for restriction fragment length polymorphic (RFLP) markers that were linked to resistance genes. Three out of the 34 RGAs were identified to be linked to the locus. Of these three markers, RGA22c was mapped to a position 3.6 cM from the *R*-locus. Polymorphic CAPS and STS markers were also detected via BSA strategy. Sequence analyses suggested that point mutations, small insertions or deletions are likely the main source of diversity of RGA clusters in this rose. Phylogenic relationships among the NBS-encoding RGAs from 12 species in five genera in Rosaceae fruit crops were evaluated. The syteny

of a genomic region that encompass powdery mildew resistance locus among *Malus*, *Prunus* and *Rosa*, which may have potential use for fruit tree disease breeding presently and gene cloning in the future (Xu *et al.* 2007).

Pathogenesis-related (*PR*) gene sequences were further isolated. Sequence comparison suggested single nucleotide polymorphisms (SNPs) in these sequences; on average one SNP occurred every 64 bp for *PR2* genes (β-1,3-glucanase) and 59 bp for *PR5* genes (osmotin). A total of 23 primers were used to genotype these SNPs for the development of single nucleotide-amplified polymorphism (SNAP) markers (Xu *et al.* 2006b). Through genetic mapping in segregating F_1 progenies, 16 out of the 23 candidate SNAP markers formed one group, and a quantitative trait locus (QTL) was detected using these markers. Minor resistance factors (QTLs) may be attributed to the contribution of *PR* genes. Candidate gene method in combination with SNAP marker is a rapid and efficient method to provide useful genetic markers for marker-assisted selection (MAS) strategy. To clone the candidate gene from Guinong No. 6, a cDNA library was constructed by subtractive suppression hybridization (SSH) method (Xu *et al.* 2006a), and the further researches are ongoing based on this library.

FUTURE CONSIDERATIONS

There is still wide scope to further investigate and explore different aspects of chestnut rose, in spite of the above mentioned advances. First of all, the genetic rules of important ornamental and agronomical traits, e.g. floral shape, color, size, and flowering time, fruit size, vitamin content, fruit production and quality, etc. require better insight, which are essential for cross-breeding. To get more cultivars adapted for diverse regions and for different uses, a schemed breeding program should be carried out. Modern biotechnology methods, e.g. transgenic transformation, embryo rescue, MAS, etc. can substantially accelerate the progress of breeding. With the union of conventional hybridization methods and bio-techniques, optimal strains for fruit or/and ornamental use may be created since preliminary investigations showed that some agronomical traits are transgressive in the first filial generation. Similarly, a strong need is also felt to further elucidate the molecular mechanism of substantially high vitamin C content in the fruits of this species, which may provide some unique clues to the synthesis and metabolism of this nutrient.

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