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Review

Molecular research and genetic engineering of resistance to Verticillium wilt in cotton: A review

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***Verticillium dahliae*, a soil-borne pathogen, causes Verticillium wilt, one of the most serious diseases in cotton, deleteriously influencing crop's production and quality. Verticillium wilt has become a major obstacle in cotton production since *Helicoverpa armigera*, the cotton bollworm, became effectively controlled in recent years. The wilt is becoming a key subject of research in cotton-resistance genetics, breeding and plant pathology. This paper reviews the recent research progress on genetic methods of resistance, the status and existing problems, traditional breeding, the main resistance mechanism, molecular markers and genetic engineering of resistance genes. It is hoped that new breeding methods and new varieties resistant to Verticillium wilt will be developed in the very near future.**

Key words: Verticillium wilt, genetics of resistance, molecular research, genetic engineering.

INTRODUCTION

Cotton (*Malvaceae gossypium* L.), an exclusive crop produced from the fibre of seeds, is very important to the world economy. During its growth process, cotton is easily subjected to diseases such as Fusarium wilt and Verticillium wilt, which can be very severe. These diseases can make the crop yellow, wilt and fall, damage the vascular tissue and ultimately cause death. Consequently, they are called the 'cancer' of cotton crops. At present, Fusarium wilt has come under control, mainly due to human efforts, whereas controlling Verticillium wilt, especially the defoliating type, has become increasingly more urgent following bollworm management of Bt cotton in recent years. The variation and differentiation of *Verticillium dahliae* strains have led to the scarcity of resistant cotton plants. In China, an area of more than 200 million hectares of cotton is subject to Verticillium wilt and the economic loss is tremendous every year (Jing et al., 1999). Thus, the need is urgent for determining the molecular mechanism and conducting genetic breeding experiments to counter defoliating Verticillium wilt.

In 1914, Verticillium wilt was first reported on upland cotton in Virginia, USA. The disease was introduced to China via imported American cotton in 1935 (Du et al., 2002). *V. dahliae* and *Verticillium albo-atrum* are the key pathogenic factors, with the former being the primary factor in China (Yao et al., 1982). The leaves and the seed of cotton can spread the disease post infection. The pathogenic fungi infect the root of the plant directly in the soil and enter the vessel through the cortical cells, in which the spores and mycelia of pathogens block the vessel of the plant. In addition, *V. dahliae* toxins and acidic glycoproteins produced by the pathogens, are also important pathogenic factors that can make the plant wilt quickly (Gan et al., 1995). *V. dahliae* in naturally infested soil exists as a highly variable form in a mixed genotypic population. Currently, no fungicides are available to cure plants once they become infected and the breeding of disease-resistant cultivars is the primary control method. Only the sea-island cotton has a higher resistance toward Verticillium wilt amongst the three cultivars of cotton (*Gossypium hirsutum* L., *Gossypium arboreum* L. and *Gossypium barbadense* L.), but cultivating it on a large scale is difficult and obtaining a new breed with high resistance character by cross-breeding it with the sea-island cotton poses a major challenge. Consequently, almost no suitable high-resistance variety to date exists in upland cotton.

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Recently, although achieving any breakthroughs has been difficult due to the absence of anti-source defoliated strains of *V. dahliae* and uncertainty regarding the molecular mechanism of resistance. Researchers have been investigating the molecular biology and genetic engineering of resistance to Verticillium wilt. Thus, elucidating the molecular mechanism would have great theoretical significance, as well as practical value, for effectively controlling defoliating Verticillium wilt and promoting sustainable development in the production of cotton.

GENETICS OF RESISTANCE TO VERTICILLIUM WILT

Regarding the inheritance of resistance to Verticillium wilt, some controversy exists as to whether the resistance is controlled by a single gene or a major gene, or is under minor quantitative trait polygene control and whether resistance is dominant or recessive. Borrow (1970) believed that the resistance to Verticillium wilt is controlled by a single dominant gene, whereas Verhalen et al., (1971) held that it is controlled by a minor quantitative trait polygene. Jiang et al. (2009) believed that the resistance of the upland cotton cultivar was controlled by two major genes with additive–dominance–epistatic effects and that the inheritance of the major gene was dominant. According to a study on the interspecific hybridisation between upland cotton and island cotton in a glasshouse or nursery, resistance was demonstrated to be controlled by a qualitative single-gene dominant or partially dominant genetic model (Wang et al., 2003). Two distinct conclusions were reached based on research of intraspecific hybridisation with upland cotton against Verticillium wilt. The first was that the resistance (tolerance) occurred via the inheritance of qualitative traits and the second was that it took place via the inheritance of quantitative traits. When the cotton plant was inoculated by single strains of Verticillium wilt in a glasshouse or in a growth cabinet, the resistance to Verticillium wilt was likely controlled by a dominant single gene. When the cotton was in the late developmental stage in the nursery, the resistance was more likely to have occurred through the inheritance of quantitative traits (Pan et al., 1994; Zhang et al., 2000).

The resistance to Verticillium wilt was demonstrated to be controlled by a single dominant gene in island cotton (Ma et al., 2000; Qi et al., 2000), while the resistance of some material is controlled by a single dominant gene and some others by two dominant genes in upland cotton (Cai et al., 2000; and Ma et al., 1999).

TRADITIONAL GENETIC BREEDING OF RESISTANCE TO VERTICILLIUM WILT

The breeding and cultivation of resistant varieties are the primary means of preventing Verticillium wilt worldwide. Traditionally, breeding for disease resistance in cotton has involved selecting resistant plants from the nursery or field among those plants suffering from serious disease. Nevertheless, this approach is not suitable for breeding seeds to generate plants resistant to Verticillium wilt. At present, the primary measure involves crossbreeding of resistant cotton (Jian and Lu, 2004).

As early as the 1960s to 1980s, in the then Union of Soviet Socialist Republics, renowned varieties having resistance to Verticillium wilt were bred, such as Tashkent 1, 2 and 3 and Andijan 6, 60 and 9070, using the Mexican half-wild cotton as a resistant resource. These varieties such as SP21 and SP37 exhibited multiple resistances and were produced in the United States in the 1970s. In the 1990s, varieties such as Acalaprema, Acala 1517-91, Delcot 344 and Acala 90, were bred in the United States, as well as varieties showing better resistance to Verticillium wilt, such as Sicala V1 and Sicala V2, in Australia (Ma et al., 1997). In China, the breeding of cotton resistant to Verticillium wilt dates back to the 1950s, with the earliest varieties, Liaomian 1 and Liaomian 2, bred by Liaoning Cotton & Flax RI. In the 1960s, two varieties resistant to Verticillium wilt were produced, Zhong 8004 and Zhong 8010. In the 1970s, varieties Shaan 1155 and Zhong 3474 with resistance to Fusarium and Verticillium wilt were amongst seven varieties bred. Shaan 1155 became the first variety to be planted on a large scale in other Shaanxi Huanghuai cotton areas by virtue of its resistance to Fusarium and Verticillium wilt. In the 1980s, 21 varieties with resistance to Verticillium wilt were bred, amongst which Zhongmian 12 became the representative variety. Amongst these 21 varieties, 20 had the trait conferring resistance toward Fusarium and Verticillium wilt. In the 1990s, varieties with improved resistance to Verticillium wilt, such as 86-6, Chuan 737, Chuan 2802, Shaan 2234, Shaan 6192, Chuanmian 239, Huai 910 and Chuanmian 243, were amongst the 111 varieties bred. Most recently, varieties having resistance to Verticillium wilt, such as Zhongzhimian 2, Shaimian 2177, Chuanmian 65, Chuanmianyou 2, Shaan 2365 and BD 18, have been created (Ma et al., 2002).

However, for a long time, no significant breakthroughs in the breeding of resistance to Verticillium wilt were achieved due largely to a lack of germplasm known to be immune or highly resistant to the fungal pathogen *V. dahliae*. Such problems may be caused directly by a lack of systematic understanding such as the pathogenetic differentiation of *V. dahliae* or the inheritance and mechanism of cotton resistance to Verticillium wilt, which made breeding plants that are resistant to Verticillium wilt very difficult (Ma et al., 2002). At present, a diagnostic variety has indicated that none of the resistant cultivars used had immunity to Verticillium wilt. The cultivars with higher resistance belong mostly to island cotton, but the susceptible plants comprised more than 70% of upland cotton and the higher resistance was less than 1% (Fang et al., 2001). The appearance of new pathotypes will account for the constant change of Verticillium wilt phy-

siological races. The resistance present in the material identified earlier will be lost with the passage of time. Therefore, the deficiency of material with high resistance has become the primary obstacle in the breeding of resistance to the disease and multiple breeding measures should be adopted actively to obtain new resistant resource material. This breeding requires time and tremendous work because of the need to hybridise amongst different varieties; for example, sometimes a wide hybridisation is needed to transfer the advantageous gene to the descendent and then backcross in multiple generations. Directional selection are adopted to make the segregated descendent become stable until it becomes isozygous (Fang et al., 2001). Achieving significant breakthroughs in the breeding of Verticillium wilt resistance will be extremely difficult if only a single means were used. Hence, breeding new varieties through a combination of multiple breeding means and the utilisation of multiple mutation breeding (e.g., chemical mutation, physical mutation and molecular mutation) is required. Also, investigation of the inheritance of resistance in detail and the standardisation of the means of identification as well as the bringing forward of explicit requests for varieties to be examined and approved should be conducted. In these ways, the process of breeding resistance to Verticillium wilt can be accelerated.

The breakdown of the disturbance of reproductive isolation can be done with the application of biotechnology. Moreover, the breeding periods have been shortened, and the breeding of disease-resistant plants has been increased by inserting only the required resistance genes into upland cotton, or recently, by the use of RNAi technology. One study has reported that the gene of higher resistance to Verticillium wilt was found in island cotton and in closely related plants. At present, the breeding of resistance to Verticillium wilt has progressed and resistant varieties have been discovered at China Agricultural University using directional transgenic At-7 of island cotton in upland cotton. Consequently, in the future, the problem of Verticillium wilt will be eradicated in China and across the world (Qi, 2006).

BIOCHEMICAL MECHANISM OF RESISTANCE TO VERTICILLIUM WILT

When cotton is infected by the wilt pathogen, the defence system begins to work, generating a series of secondary reactions. Three aspects are involved in the mechanism of resistance. These are tissue resistance, physiological and biochemical resistance and micro-organism resistance.

Tissue resistance

When the root of cotton seedlings becomes infected by the fungus, the tissue structure changes, colloids are produced and the pathogens are wrapped by colloids. The infected cells around the vessel are activated and restricted inside the vessel by the synthesis of lignin Denning and terpenoids; cambium activity is accelerated and a new vessel is formed. In this process, with the accumulation of lignin produced in secondary xylem, the mechanical strength of the stem is enhanced and the cellulose avoids chemical and biological degradation. The increased lignin makes the tissue tighter and decreases the intercellular space, which not only increases the capability of resisting disease but also prevents the infection of fungus and internal spread (Smit and Dubery, 1997).

Physiological and biochemical resistance

The proliferation and infection of pathogens is prevented by a series of antimicrobial compounds synthesised in cotton, such as phytoalexins, enzymes, pathogenesis-related proteins (PRPs) and antimicrobial peptides (Zhou, 2006).

Phytoalexin synthesis

Many plants secrete and accumulate phytoalexin to resist infection by fungi and bacteria. The five antimicrobial compounds, gossypol (G), hemigossypol (HG), 6-methoxyhemigossypol (MHG), deoxylhemigossypol (dHG) and desoxy-6-methyoxyhem igossypol (dMHG), are associated with resistance to Verticillium wilt. HG, MHG, dHG and dMHG have been found to have a strong toxicity toward *V. dahliae* in liquid nutrient media and can rapidly kill all conidia and mycelia in low concentrations at the same time, which can inhibit spore germination (Mace et. al., 1985). Bell (1967) believed that the phytoalexin formation rate indirectly influences the formation and metabolism of other substances within resistance organisations. Jelly is not disassembled in xylem when the concentrations of pectinase and cellulase in resistant varieties are lower than those concentrations in the pathological varieties because the secretion and synthesis of fungal hydrolytic enzymes inhibited, accounted for the rapid synthesis of phytoalexin.

Enzymes and proteins

Many plants prevent the infection of pathogens using their own proteases. This process is primarily through regulating the antioxidant, anti-hydrolysis, synthesis of phytoalexin and synthesis of new wall systems of the plant. These proteases include glutathione S-transferases (GSTs); superoxidase dismutase (SOD); peroxisome (POD); polyphenol oxidase (PPO); glucose oxidase (GO); lipid transfer protein (LP); the protease inhibitor (Polygalacturonase inhibiting proteins, PGIPs et al.),

hydrolase (chitinase, 1,3-β-galactosidase and L-phenylalanin ammonialyase (PLA)); (+)-δ-cadinene synthase (CAD); farnesyl diphosphate synthase (FPPS); isopentenyl diphosphate isomerase (IPI); 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and cell wall proteins.

Impact of other chemical substances

The soluble sugar Denning and alanine contents are associated with resistance. Gan et al., (1995) reported that the sugar content of resistant and susceptible strains was lower than that of a non-inoculated healthy cultivar. The Denning content of resistant varieties is higher than that of the susceptible strain. In the same cultivar, the Denning content increases as the disease becomes more severe. Bell (1969) reported that the formation rate of Denning in sea-island cotton is faster (about 24 h) than in the upland cotton, when the stem of sea-island cotton is infested. Denning has a strong effect on the anti-formation of spores (Beckman, 1966; Bell, 1969).

Zhu et al., (2007) reported that the percentage inhibition of China Sumac, Eupolyphaga seu Steleophaga, Common cnidium fruit, Nightshade, Herba Ephedrae, Croton fruit, Amur Corktree Bark and Shortsepal Goldthread by *V. dahliae* is over 45%. Further study revealed that China Sumac, Eupolyphaga seu Steleophaga and Common Cnidium fruit have the best rates of inhibition on *V. dahliae* (Zhu et al., 2007).

Plant antimicrobial peptides

Antimicrobial peptides from other plant extracts or synthetic peptides can resist Verticillium wilt through the inhibition of amylase activity or through a series of rapid responses including raising the K^+ outflow, Ca^{2+} absorption, changing membrane permeation and ion flow in different binding sites of the lipid bilayer on the fungal cell membrane (Yang et al., 1997). Viridin, a new extracellular antifungal peptide with a yield of 10 mg/L, has been isolated from the culture medium of the mould *Trichoderma viride*. This protein inhibits the growth of the cotton pathogen *V. dahliae* with an IC50 of 6 μM (Hao et al., 1999). Alfalfa antifungal peptide (alfAFP) is a plant defensin isolated from seeds of *Medicago sativa* that displays strong activity against the agronomically important fungal pathogen *V. dahlia.* The expression of alfAFP in transgenic potato plants provides robust resistance against the pathogen (Zhang et al., 2008). Transgenic cotton plants expressing the synthetic antimicrobial peptide D4E1 have been produced through *Agrobacterium*-mediated transformation. *In vitro* assays with crude leaf protein extracts from T0 and T1 plants have confirmed that D4E1 was expressed at levels sufficient to inhibit the growth of *Fusarium verticillioides* and *V. dahliae* compared to extracts from negative control plants transformed with pBI-d35S(omega)-uidA-nos (CGUS) (Rajasekaran et al., 2005).

Interaction with other micro-organisms

The resistance mechanisms of cotton can be induced by some micro-organisms. The primary reason is that the host's metabolism is affected, including the formation of chemical and biological barriers at the fungal infection site. To date, the plant growth promoting rhizobacteria CS85, endophytic bacterial strains XJUL-6 from *Urtica cannabina* L. in XinJiang, *Bacillus subtilis* N1729 and *Paenibacillus* LC105 have all been reported to show strong resistance to *Fusarium oxysporum* and *V. dahlia* (Wang et al., 2004; Chen et al., 2007; Zhang et al., 2007; Zhou et al., 2007).

MOLECULAR RESEARCH ON THE RESISTANCE TO VERTICILLIUM WILT

With the development and application of molecular boilogy techniques, Verticillium wilt resistance research has reached the molecular level. Molecular markers have been a valuable tool in cotton breeding investigations. Various marker techniques used in cotton research include random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs) and quantitative trait loci (QTLs).

Molecular markers

RAPD

RAPD has an important role in the resistance of cotton to Verticillium wilt. Sequences were determined for codominant RAPD markers closely linked to the Ve locus, a dominant Verticillium wilt resistance gene, in tomato by Kawchuk et al., (1998). High-resolution linkage analysis using F_2 progeny segregating for resistance and marker-assisted selection indicated that the linkage between the genetic markers and the Ve locus is less than 0.67 ± 0.49 cm (Kawchuk et al., 1998). SV-M1 and SV-M3 markers were found to be linked closely with the major resistance gene, VWR1, in the cross of Sicala V-1 (R) and Siokra 1-4 (S) by AFLP (Lyon, 1999). DNA fingerprinting of 25 upland cotton cultivars resistant or susceptible to *V. dahliae* was conducted using 26 primer amplification products that were selected from 40 that appeared as polymorphisms in the DNA amplification of 25 cotton varieties; their genetic diversity was also analysed (Guo et al., 1999). The genetic authenticity of China's existing Verticillium-resistant (tolerant) varieties (lines) was revealed based on DNA analyses. Fang et al. (2001) found

a RAPD marker with OPB-191300 linked to wilt resistance in cotton. The exchange and genetic distance of OPB-191300 with Verticillium wilt resistance were 12.1% and 12.4 cm, respectively. Zhang et al., (2002) reported that 15 random primers were used to amplify the total DNA of 58 glandless cotton varieties. Amongst the total 106 RAPD bands identified, 50 bands (47.2%) were polymorphic and the varieties were classified into six RAPD groups (Zhang et al., 2002).

SSRs

Ma's group reported that 175 F_2 individuals developed by a cross of *G. barbadense* α15-3493×*G. hirsutum* Shihezi 875 were employed for a molecular marker population. The distance between a locus related to Verticillium wilt resistance and the SSR marker BNL3556 was 13.1 cm, and this locus accounted for 50.1% of the phenotypic variance. It was a major gene locus (Du et al., 2004). F_2 individuals (182) from the cross between the susceptible *G. hirsutum* CCRI 8 and the resistant *G. barbadense* Pima 90-53 were exploited to detect the SSR molecular marker linked with the gene of Verticillium wilt resistance. Based on the resistance of F_2 plants, the DNA of 10 resistant and 10 susceptible plants were used to construct resistant and susceptible gene pools, respectively. The pools were screened with 768 pairs of SSR primers, of which primers BNL2440 and BNL3255 showed polymerphisms between resistant and susceptible DNA pools and the parents also had the same polymorphic markers on the same locus. A polymorphic fragment of 208 bp amplified by primer BNL3255 was designated as BNL 3255-208. The genetic distance between the locus of Verticillium wilt resistance and the BNL3255-208 marker was 13.7 cm. The BNL3255 marker was located on the short arm of chromosome 5 (Zhen et al., 2006). To verify the BNL3255-208 marker linked with the gene of Verticillium wilt resistance in Pima 90-53, 100 individuals and 131 individuals of two newly derived F_2 populations were used from the crosses between the susceptible CCRI8 or Han 208 and the resistant *G. barbadense* variety Pima 90-53, respectively. The identification of disease resistance in F_2 populations was performed in a growth chamber. Of 79 resistant plants, 70 (88.6%) were identified as having the BNL3255-208 marker in the population from CCRI8 and Pima 90-53. Also, for the population of Han 208 and Pima 90-53, 85 of 100 resistant plants had the BNL3255-208 marker. The BNL3255-208 marker was recovered and re-amplified and then cloned and sequenced. The marker consisted of a fragment of 211 bp, including 10 repeats of TG (Wang et al., 2007).

AFLP

In 1999, Mona first used AFLP markers to mark resistance genes. Qi et al., (2000) found a susceptible gene fragment and four resistance-related DNA fragments whose lengths were about 200–525 bp. Using two pairs of primers to amplify the DNA fragments, he obtained two specific bands, one associated with resistance (292 bp), the other with susceptibility (410 bp). The distance between the resistance gene marker and the resistance gene was 20.947 cm and the distance between the susceptible gene marker and resistance gene was 13.49 cm. Zhu et al. (2001) used an AFLP marker as the accessorial selection in the process of Verticillium wilt resistance of upland cotton and found that the distance between the resistance gene marker and the resistance gene was 9.29 cm. Wang et al. (2004) reported similar genetic diversity between the cotton cultivars from Huanghe valley and those from Changjiang valley by AFLP marker experiments. These markers lay a foundation for marker-assisted selection of disease resistance.

QTL

Both Gao et al., (2003) and Zan et. al., (2008) believed that the resistance of Verticillium wilt in F_2 individuals of a cross of *G. hirsutum* cv. Handan 208 (R) × *G. barbadense* cv. Pima 90 (S) is controlled by two major QTLs and one minor QTL. Yang et. al., (2007, 2009) reported in detail the QTL mapping of genes resistant to Verticillium wilt in *G. barbadense* and in *G. hirsutum*, which might lay the foundation for resistance to Verticillium wilt and could promote effective cotton breeding.

QTL mapping for resistance to Verticillium wilt in *G. barbadense*: A strain highly tolerant to Verticillium wilt, *G. barbadense* var. Hai 7124 and one highly sensitive to Verticillium wilt, *G. hirsutum* var. Junmian 1, were crossed. The F_1 seeds produced self-pollinated F_2 progeny comprising 128 individuals and BC_1 was produced from a cross between (Hai 7124 × junmian l) × Junmian 1. The F_2 individuals were inoculated with a non-defoliating isolate, BP2 and the disease grades were investigated at the seedling and mature stages. QTL mapping and an estimation of their genetic effects were performed according to the method of combination interval mapping (CIM). Four QTLs were detected and located on chromosome A5, A7 and A8 at the seedling stage; three QTLs were detected and located on chromosome A5, A7 and A9 at the mature stage. The contributions of these QTLs to the phenotype ranged from 8.0 to 17.1%. The isolate, BP2 and two defoliating isolates, VD8 and 592, were used to inoculate the BC_1S_2 families. The resistant QTL was detected using the CIM method (at the seedling and mature stages, respectively). In the BP2 nursery, one QTL was detected at the seedling stage and two at maturity, located on chromosomes D4, A8 and D4. In VD8 nursery, two QTLs were detected on A5 and A8 and three QTLs located on A5, D5 and D11. In the 592 nursery, three QTLs were located on A5 and D5 and two were

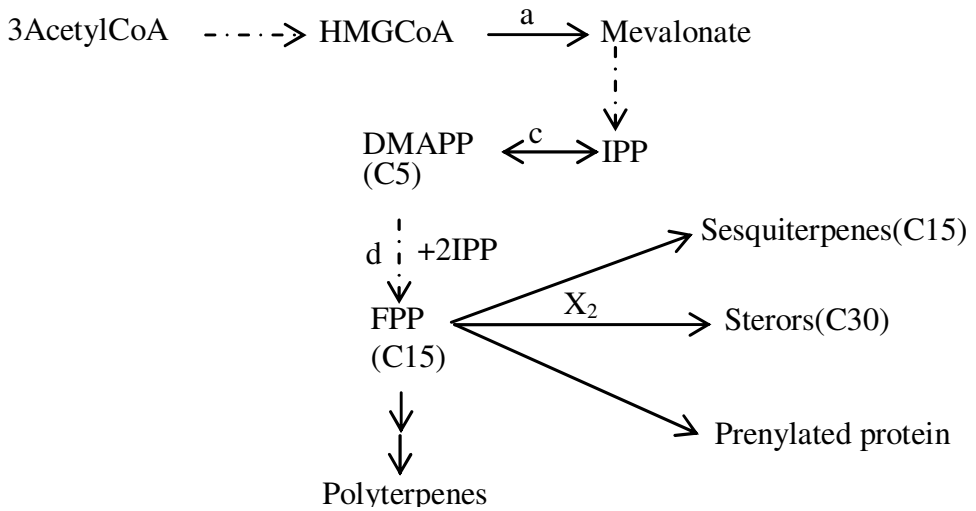

Figure 1. Biosynthesis of sesquiterpenes in plants.

Note: Acetyl-CoA, acetyl coenzyme A; HMGCoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isoprenyl pyrophasphate; DMAPP, demethyacral pyrophasphate; FPP, farnesyl pyrophosphate; a, HMGCoA reductase. b, Plastidic 1-deoxy-Dxylulose-5-phosphate pathway. c, Isopentenyl pyrophosphate isomerase. d, Prenyl transferase (from Gaffe et al., 2000).

located on D5 and D11. These QTLs accounted for 7.8 to 22.3% of the phenotypic variation. These results suggested that different resistance QTLs are involved for different isolates at different stages in *G. barbadense* (Yang, 2007, 2009; Bolek et al., 2005; Yang et al., 2008).

QTL mapping for resistance to Verticillium wilt in *G. hirsutum*: The Verticillium wilt-resistant *G. hirsutum* line 5026 and the highly sensitive *G. hirsutum* var. Li8 were crossed. The F_1 seeds produced self-pollinated F_2 progeny that produced self-pollinated $F_{2:3}$ progeny comprising 154 lines. A recombinant inbred line (RIL) population comprising 169 lines was produced, derived from the cross of 5026 and Li8. The $F_{2:3}$ lines were inoculated with isolate VD8, and the RIL lines were separated into three parts and inoculated with isolates BP2, VD8 and 592, respectively. The disease reaction was investigated in each nursery at the seedling and mature stages. The resistance inheritance models were analysed using the major gene and minor gene mixed model system in the two populations. The result suggested that the trait of resistance to Verticillium wilt is is controlled by two major genes in *G. hirsutum*. The resistance QTLs were mapped in $F_{2:3}$ families using the CIM method. Three QTLs were detected at the seedling stage and located on the LG01 linkage group and chromosomes D8 and D7; four QTLs were detected at the mature stage and located on the LG01 linkage and chromosomes A11, D8 and D7. These QTLs explain 6.39 to 33.22% of the variation of phenoltype. The resistant QTLs were mapped using the RIL population. In the BP2 nursery, three QTLs were detected at the seedling stage and one QTL at the mature stage and they were located on chromosomes A5, D6, D9/D10 and D11. In the VD8 nursery, two and one QTLs were detected at the seedling and mature stages and they were located on chromosomes A11, A8 and D9/D10. In the 592 nursery, three and one QTLs were detected at the seedling and mature stages and they were located on chromosomes A5, A11/D11, A8 and A5. These QTLs explain 5.36 to 11.8% of the phenotypic variation (Yang et. al., 2007, 2009). Although the genes conferring resistance to Verticillium wilt have not yet been cloned, the molecular map and gene locations will form the basis of the gene cloning and cultivation of resistant varieties.

Isolation and cloning of Verticillium wilt-resistance genes

Research on resistance-related genes

The research domain of resistance-related genes includes genes related to phytoalexin synthesis, pathogenesis-related protein genes and defence protein genes related to plant structure and pathogenic factor detoxification genes.

Genes related to phytoalexin synthesis: Sesquiterpene is an important antitoxin and its synthesis pathway is depicted in Figure 1 (Gaffe et al., 2000; Meded, 2001). Phytoalexin biosynthesis occurred earlier in the resistant cotton cultivar sea-island than in the susceptible cotton cultivar after inoculation with a defoliating isolate of the pathogen *V. dahliae*. Some genes related to gossypol biosynthesis have been found and cloned, including CAD, FPPS, IPI and HMGCR. All of these become the

key regulatory enzymes in phytoalexin synthesis, which can kill the pathogens through increased local concentrations in the plant.

Pathogenesis-related protein genes: The pathogenesis-related (PR) protein, which not only accumulates at the site of infection, but also in the whole plant, inhibits the infection by pathogens. The PR genes include lipid transfer proteins, chitinase, β-1, 3 glucanase, non-expressor of pathogenesis-related genes 1 (GhNPR1), thionin, gastrodia antifungal protein and lectin-like protein. Chitinase and β-1,3-glucanase are two important factors of resistance. Once transgenic cotton is infected by pathogens, it will rapidly produce a lot of chitinase and glucanase, which could degrade fungi cell walls, thereby producing the resistance (tolerance) (Cheng et al., 2005). GhNPR1 was found to be induced by signalling molecules for plant defence responses, such as methyl jasmonate, salicylic acid and ethylene, as well as upon attack by pathogens such as *F. oxysporum* and *Xanthomonas campestris.* These results suggest that GhNPR1 may play an important role in the response to pathogen infections in cotton plants (Zhang et al., 2008).

Defence protein genes related to plant structure: Lignifications in the infected cell of the plant have become an effective means to slow down the spread of pathogens. The defence protein genes include PAL, cinnamate-4-hydroxylase (C4H), POD, polygalacturonase-inhibiting proteins (PGIPs) and laccase. PAL and C4H also accumulate in cells adjacent to metaxylem and are presumably involved in maintaining a supply of phenylpropanoid precursors to the enucleated xylem for further lignin synthesis. The cationic peroxidase accumulates in the xylem at sites of secondary thickening and in the middle lamella. The three proteins are also involved in defensive lignifications (Colin et al., 1994). PGIPs are ubiquitous plant cell wall proteins that are directed against fungal polygalacturonases (PGs), which are important pathogenicity factors. The inhibiting activity of PGIPs directly reduces the aggressive potential of PGs, and in addition, causes PGs to form more long-chain oligogalacturonides that are able to induce defence responses, thereby indirectly contributing to plant defence (Huang et al., 2008). Conclusive evidence of the occurrence of laccase in a tissue must demonstrate that the enzyme is able to oxidise quinol with the concomitant uptake of oxygen. Laccase is involved in the pigmentation process of fungal spores, the regeneration of tobacco protoplasts, as fungal virulence factors and in lignification of cell walls and delignification during white rot of wood (Wang et al., 2008).

Pathogenic factor detoxification genes: Oxidative stress occurs when the level of prooxidants exceeds the level of antioxidants in cells, resulting in the oxidation of cellular components and consequent loss of cellular function. The production of hydrogen peroxide (H_2O_2) and antioxidants in some plants is one of the molecular mechanisms for regulating the function of resistance. Glucose oxidase (GO) catalyses the oxidation of β-D-glucose to gluconic acid by utilising molecular oxygen as an electron acceptor with the simultaneous production of H_2O_2. When the GO gene was transducted in cotton, the cotton plant became a dwarf, the boll stems became longer, the number of bolls increased and the fibre attributes improved. The GO gene is a wide-spectrum resistance gene and it would be important in the breeding of Verticillium wilt resistance in cotton (Liu et al., 2003). The methionine sulphoxide reductase (Msr) protein plays key roles in the resistance to pathogens. The oxidation of methionine (Met) to methionine sulphoxide [Met (O)] results in a decrease or loss of the biological activity of related proteins. A study found that peptide methionine sulphoxide reductase (msrA) can reduce Met (O) to Met, thus restoring the biological function of the oxidised proteins (Zhao, 2004). Novel features of nonsymbiotic haemoglobin gene 1 (GhHb1) have also been identified, indicating that GhHb1 expression is activated in the cotton roots after inoculation with *V. dahliae* and that exogenous H_2O_2 induces GhHb1 expression. These results suggest that GhHb1 may play a role in the defence response of *G. hirsutum* against *V. dahliae* invasion (Qu, 2005, 2006).

Isolation and cloning of resistance genes

Resistance gene analogues (RGAs) and defence gene analogues (DGAs): The majority of plant disease-resistance genes (R genes) isolated, encode a predicted nucleotide binding site (NBS) domain. The NBS sequences can be divided into two major groups. Group I NBS domains contain TIR (Drosophila Toll or human interleukin receptor like) and Group II does not. The two groups are distributed amongst dicot species. NBS domains related to R genes show a highly conserved amino acid backbone, making it possible to isolate RGAs by polymerase chain reaction (PCR) with degenerate primers. Tu et al. (2003) designed multiple combinations of primers from two conserved motifs in the NBS regions of R genes of various plants to amplify genomic DNA of sea-island cotton var. Pima 90 (*G. barbadense*). The desired bands were purified from the gel and then cloned by T/A cloning. After sequencing and alignment, 31 RGAs were isolated. Amongst the RGAs, 19 were uninterrupted open reading frames (ORFs). The 31 RGAs, as well as 34 RGAs of (*G. hirsutum*) germline M 249 obtained by searching GenBank were divided into two classes. Class I included only upland cotton RGAs, and class II included all upland cotton and sea-island cotton RGAs. Two distinct groups, the TIR type and non-TIR type, were classified by phylogenetic analysis in sea-island cotton. The analysis of the 19 ORF structures suggested that they contain the motifs such as a P loop, Kin 2, 'PLAL' and RNBS A, B and C, as defined by Meyers (1999). This

Table 1. Major genes on genetic engineering of cotton resistance.

Protein/Gene	Protein Properties	Mr(kDa)	Mechanism
Lipid transfer protein, At-7	PR	9 - 24	Accelerate the intermembrane transfer of glycolipids *in vitro*
Polygalacturonase inhibiting proteins, PGIPs	Cell wall synthesis enzyme	37.0	Against fungal polygalacturonases
Glucose oxidase, GO	Detoxification enzymes	140	H_2O_2 produced can kill the pathogen, active the defense system
Chitinase, Chi	PR	25 - 35	Hydrolysis N-acetyl-glucosamine β-1, 4 bond
β-1,3Glucanase	PR	33 - 41	Hydrolysis β-1, 3 glucanase
Thionin	PR	5	Through electrostatic interaction of fungal cell membrane phospholipids to make membrane produce hole, lead the pathogen to death
Ricin	Ribosome-inactivating protein	30 - 70	Modify AMP of ribosomal 28SrRNA No. 4324, impede the synthesis of protein
Antifungal protein 1, Rs-AFP1	PR	5685	Kill a broad spectrum of micro-organisms
Gastrodia antifungal protein GAFP	PR	10 - 14	Acting on the cell walls of hypha
D4E1	PR	1.1	Inhibited extensive colonization and spread by the fungus in cotyledons and seed coats
Nonrace-specific disease resistance, NDR1	PR	30	Signal transduction of defensive reaction in plant induced
None expresser of PR gene, NPR1	PR	65	Signal transduction of defensive reaction in plant induced

indicated that NBS-type disease-resistance gene analogues in sea-island cotton, share a common origin and evolution with the other plants (Tu et al., 2003). In another study, Gao, (2006) cloned 79 NBS sequences, 21 STK sequences and 11 DGAs from sea-island cotton variety Hai 7124 (R). The phylogenetic analysis of the 79 NBS-RGAs and NBS-RGA nucleotide sequences of cotton already deposited in GenBank identified one new sub-cluster. The deduced amino acid sequences of NBS-RGAs and STK-RGAs were divided into two distinct groups: the TIR group and non-TIR group, and an A group and B group, respectively. The expression of RGAs and DGAs having consecutive ORFs was also investigated and six NBS-RGAs and one STK-RGA were found to be induced. One DGA was upregulated by infection of *V. dahliae* strain VD8. Four TIR-NBS-RGAs and four non-TIR-NBS-RGAs were arbitrarily used as probes for Southern blotting of which 2–10 blotted bands were detected. In addition, since three non-TIR-NBS-RGAs exhibited the same hybridisation patterns, Gao, (2006) conjectured that these three RGAs form a clustered distribution in the genome. These RGAs and DGAs can also serve as potential probes for the identification and cloning of R genes from the cotton genomic library.

Resistance gene cloning: To date, using suppression subtractive hybridization (SSH), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and cDNA-AFLP, researchers have obtained the following resistance-related genes: FPPS and CAD (Liu et al., 1999), MsrA (Zhao, 2004), the pathogenesis-related 10 family of *G. hirsutum* (PR 10-5, PR 10-11, PR 10-12, PR 10-16) and disease resistance-responsive family protein (Zhu, 2006), PR8, PR18, serine/threonine kinase, glutathione S-transferase, alcohol dehydrogenase, cytochrome P450, ACC oxidase (Wang et al., 2008), GhHb1 (Qu et al., 2005, 2006), tau glutathione S-transferase subunit, GhGa, GhDAHPS, GbSTK, GhCCoAOMT, GhLac, GhCyP (Ma et al., 2008), GhNPR1 (Zhang et al., 2008) and IPP (Wang et al., 2009).

GENETIC ENGINEERING OF RESISTANCE TO VERTICILLIUM WILT

Verticillium wilt is a worldwide destructive cotton disease, especially for upland cotton, which comprises the largest planting area amongst cotton cultivars. The disease incidence has increased in recent years due mainly to the increasing use of susceptible varieties. The breeding of varieties highly tolerant or resistant to Verticillium wilt has been confirmed to be the most effective disease control method. For decades, researchers have investigated the genetics of resistance, biochemical mechanism, genetic breeding and cloning of resistance genes and have made significant progress in obtaining resistant cultivars. With the recent advances in molecular biotechnology, especially molecular marker technology, many researchers have tried to use molecular markers to locate resistance genes. Those molecular markers linked closely with the resistance genes have been utilised in molecular marker-

assisted selections (MAS), which will comprehensively promote the process of resistance breeding in cotton. The identification of molecular markers associated with R genes can greatly facilitate their cloning.

Many resistance genes have been applied to genetic engineering (Table 1), such as chitinase, β-1, 3 glucanase, thionin and glucose oxidase. Currently, the most successful case is that use by Qi (2006) who breded cotton plant, which has the trait of high resistance to Verticillium wilt. In his research, he obtained a resistance-related gene, At-7, using the endotoxin of virulent strain 'V991' to infect the island cotton and then transferred it to upland cotton. The disease incidence of the transgenic plant was controlled in the range of 22.58 to 55.81% and the disease index range was 7.26 to 19.08%. Meanwhile, the plants displayed good boll and high-yield features.

In the future, an adaptation of a combination of conventional breeding and modern biotechnology methods in agriculture to achieve major breakthroughs in cotton breeding of disease resistance should be applied. The main techniques for germplasm enhancement have involved multiple crosses, interspecific crossing and gene/ DNA transformation such as *Agrobacterium*-mediated, particle gun bombardment and pollen tube pathway and physical irradiation inducement.

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