Genetics and Biochemistry of Insect Resistance in Maize

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Abstract Insects are a major concern for maize production worldwide. Host plant resistance to insects involves a number of chemical and biochemical factors that limit, but rarely eliminate insect damage. Most chemical and many biochemical factors involved in resistance to insects are synthesized independent of the pest as phytoanticipines. These factors are stored in sequestered forms that are modified to active structures upon insect infestation and tissue damage. Because the genetic basis of varietal responses to insects for maize is quantitative in nature, quantitative trait locus analysis has been a standard approach to describe insect resistance. These studies often examined correlated biochemical traits to link genetic loci with biological mechanisms. Recently there has been a realization of the importance of herbivore enemies that are attracted by maize in response to herbivore damage. Upon infestation, maize releases volatile chemicals to actively recruit parasitic wasps or nematodes to combat the insect pests. In this review we examine the current state of knowledge of the biochemical, genetic and plant-insect tritrophic mechanisms involved in maize resistance to insect pests.

1 Introduction

Maize, like all other crop species, suffers damage from a large number of insect pests (Dicke, 1977). Insects are a particularly acute problem for maize production in tropical regions. Genetic differences in the host plant response of maize varieties to insects are almost exclusively quantitative in nature. In this review we will summaries the current state of knowledge of maize response to insect attack in three different areas, biochemical basis of resistance, genetic basis of resistance and the rapidly expanding knowledge on maize-insect tritrophic interactions. We will limit ourselves to native plant resistance and leave the extensive topic of transgenic resistance mediated by the expression of foreign proteins to other authors of these volumes (Vol. 2, Chap. 3).

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2 Biochemistry of Resistance

2.1 Chemical Defense

Plants produce hundreds of thousands of unique low-mass natural products, known as secondary metabolites. Secondary metabolites are distinct from components of primary metabolism in that they are generally non-essential for basic metabolic processes of the plant, but improve defense against microbial attack, herbivore predation and control allelopathic interactions. A common feature of many of these compounds is that they have a chronic rather than an acute toxicity on insects, and their effects are less dramatic than those of the synthetic insecticides. Maize host-plant resistance to corn earworm (*Helicoverpa zea*) (CEW) has been attributed to the presence of the secondary metabolites C-glycosyl flavone maysin (2”-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl) luteolin) and the phenylpropanoid product chlorogenic acid in silk (see Sect. 3.3). The first brood of the European cornborer (*Ostrinia nubilalis*) (ECB) is controlled by high levels of the benzoxazinoid DIMBOA in seedlings and young plants (Klun et al., 1970). These secondary metabolites are produced independent of the presence of the pest in a tissue- and developmental-specific manner. In addition to secondary metabolites, ubiquitous phenolic acids, especially ferulic acid, may contribute to insect resistance in maize.

The biosynthesis of these defense-related metabolites has a common root in the shikimic acid pathway (Fig. 1). Maysin, chlorogenic acid and phenolic acids originate from the phenylalanine branch of the pathway, biosynthesis of benzoxazinoids shares intermediates with the tryptophan metabolism.

2.1.1 Benzoxazinoids

Natural benzoxazinoids were discovered in 1960 in rye when resistance against fungi was investigated (Virtanen and Hietala, 1960). Benzoxazinoids have been predominantly found in genera of the Gramineae. Outside the grasses, they have been isolated from several species of the Acanthaceae, Ranunculaceae and Scrophulariaceae. In rye and wild barley species DIBOA (2,4-dihydroxy-2*H*-1, 4-benzoxazin-3(4*H*)-one) is the predominant aglucone moiety, in maize and wheat it is the methoxy derivative DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) that prevails (Fig. 1). Benzoxazinoids are stored as D-glucosides. The glucosides are compounds of low toxicity that can undergo enzymatic and chemical degradation. In the intact plant cell, benzoxazinoid-glucosides and the specific ß-glucosidase are kept in two different compartments, the vacuole and the plastid, respectively (Babcock and Esen, 1994). In maize the enzymatic release of the aglucone by the ß-glucosidase after wounding followed by disintegration of the cell compartments, is completed within half an hour. In the case of benzoxazinoid synthesis, plants implement the concept of phytoanticipines: biosynthesis is independent of the presence of the pest, an alleviated product is stored and activated

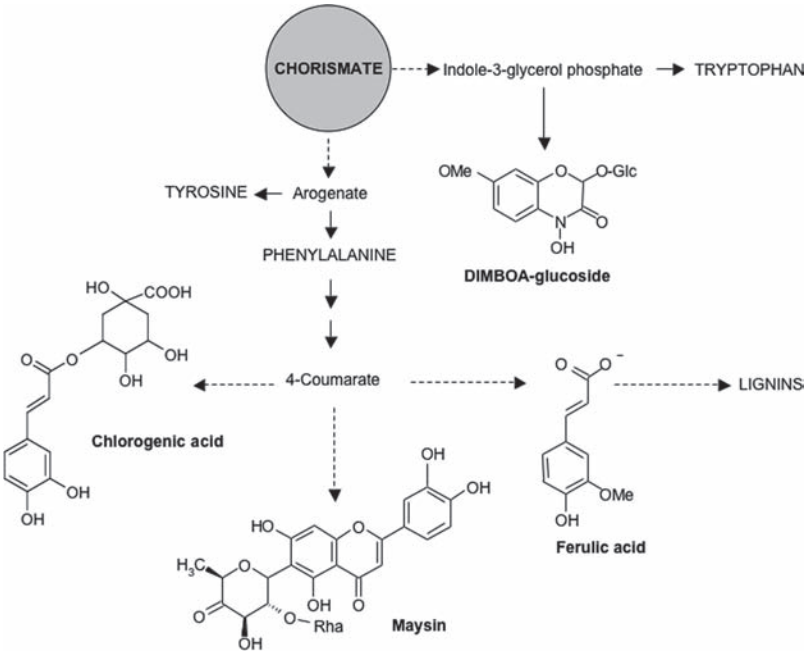

Fig. 1 Biosynthetic relationship between defense-related maize metabolities. Chorismate, synthesized by the shikimic acid pathway is the central intermediate. Chlorogenic acid, maysin and ferulic acid share the steps of the phenylpropanoid pathway to 4-coumarate and the tryptophan branch of aromatic acid biosynthesis. Ferulic acid is a general intermediate of the lignin biosynthesis, a primary biosynthetic pathway. Chlorogenic acid, maysin and DIMBOA are secondary metabolites. The primary metabolites are given in capitals

following infestation. DIMBOA-glucoside content in corn seedlings can reach concentrations up to 10 mmoles per kg of fresh weight (Long et al., 1975).

Benzoxazinoids control a wide range of pathogens and pests (bacteria, fungi, and insects) due to the general toxicity of the compound. DIMBOA is an enzyme inhibitor of α-chymotrypsin (Cuevas et al., 1990), aphid cholinesterase (Cuevas and Niemeyer, 1993) and plasma membrane H^+ -ATPase (Friebe et al., 1997). The hemiacetal DIMBOA undergoes an oxo-cyclo tautomerization. The aldehyde group of the oxo-form reacts with the ε-NH2 group of N-α-acetyl lysine, a model substrate for lysine residues in proteins (Perez and Niemeyer, 1989). The 7-MeO-group facilitates N-O bond heterolysis and hence the formation of a reactive, multicentered cationic electrophile (Hashimoto and Shudo, 1996). The presence of 7-MeO group might open yet another mode of action: it has been demonstrated that dehydration of DIMBOA generates a reactive formyl donor towards $-NH_2$, -OH, and -SH groups (Hofmann and Sicker, 1999).

The correlation between DIMBOA content and protection against insect feeding damage was assayed by two approaches: larval development and insect performance were analyzed on DIMBOA diets (Campos et al., 1989) and insect damage was monitored on high and low DIMBOA cultivars (Barry et al., 1994). These investigations demonstrated that DIMBOA can act as feeding deterrent and reduce the viability of insect larvae. The genetic basis of this resistance was demonstrated and the DIMBOA content was successively elevated in breeding programs (Klun et al.,, 1970, Grombacher et al., 1989). A *benzoxazinless* mutant defective in DIMBOA-biosynthesis was isolated in 1964 by Hamilton. The respective gene, *Bx1* was molecularly identified by directed transposon tagging using the *Mutator* transposon system of maize (Frey et al., 1997). *Bx1* is homologous to the alpha-subunit of tryptophan synthase and catalyzes the formation of indole. The sequence and gene structure of *Bx1* proves that this branchpoint gene of benzoxazinoid biosynthesis has its evolutionary origin in a duplication and subsequent modification of the *TSA* gene of the primary metabolism. In a separate study, BX1 enzyme was given the name indole synthase (Melanson et al., 1997). The introduction of four oxygen atoms into the indole moiety that yields DIBOA is catalyzed by four cytochrome P450-dependent monooxygenases *Bx2* through *Bx5* (see Vol 2, Genes and Gene Families, 3. P450s), a further oxygen function is introduced by *Bx6*, a 2-oxoglutarate dependent dioxygenase (Frey et al., 2003). Biosynthesis of the aglucone entity is completed by the *O*-methyltransferase *Bx7*. Two glucosyltransferases, *Bx8* and *Bx9* (von Rad et al., 2001) function to detoxify DIMBOA. All DIMBOA-glucoside biosynthesis genes are located on the short arm of chromosome 4, *Bx1* to *Bx6* and *Bx8* map within 6 cM. These biosynthetic genes may account for the major QTL for first brood ECB resistance at this position. Orthologs to the maize genes *Bx1* to *Bx5* have been isolated from wheat and a wild barley species (Nomura et al., 2003, 2005; Grün et al., 2005), and phylogenetic analysis reveals that benzoxazinoid biosynthesis in the grasses has a monophyletic origin. Regulatory genes of the pathway have not been identified.

2.1.2 Phenolic Acids and Cell Wall Components

Plants contain significant quantities of various polyphenolic acids, as well as their glycosides and esters. These compounds are implicated in two defense concepts, the phenolic fortification of cell walls and the deterrent effect of fiber content. Main components that strengthen the cell wall as mechanical barrier are (*E*)-ferulic and (*E*)-*p*-coumaric acid which are attached to hemicellulose through pentose sugars. Dimers of these bound phenolic acids can be generated enzymatically by peroxidase (5, 5'-diferulic acid) or through photochemical reactions (truxillic and trucinic acids). Formation of such dimers may increase the mechanical strength of the cell wall by the cross-linking of hemicellulose and hence reduce degradability (Bergvinson et al., 1995). Free phenols, mainly 4-coumaric and ferulic acid, were implicated as factors contributing to resistance of maize against ECB and the maize weevil (*Sitophilus zeamais*), and recently, to pink stalk borer

(*Sesamia nonagrioides*) (Santiago et al., 2006). Interestingly, the transgenic expression of wheat oxalate oxidase in maize significantly increased the phenolic concentrations. The highest increase was detected for ferulic acid. Field testing showed that the transgenic maize exhibited more resistance to ECB than the non-transgenic counterpart. Since a negative correlation between ferulic acid concentration in meridic diets and larval growth rate was found, it may be speculated that ferulic acid content is a main factor of resistance for these plants. The level of DIMBOA was decreased in the transgenic plant, possibly due to diversion of metabolism to the phenolics (Fig. 1). It is suggested that transgenic oxalate oxidase elicits defense responses by generation of H_2O_2 and activating jasmonic acid signaling (Mao et al., 2007).

The phenolic acid esters chlorogenic acid and the C-glycosyl flavone maysin have been implicated in CEW antibiosis (Sect. 3.3, Fig. 1). Both compounds were found to be feeding deterrents. For chlorogenic acid an inhibition of development and severe impairment of larval growth was demonstrated in feeding studies with *Spodoptera litura*. Foliar enzymes such as polyphenol oxidases and peroxidases increase the inhibitory effect by generation of chlorogenoquinone which in turn alkylates dietary protein and reduces its nutritional value. Covalent binding of ubiquinones to nucleophilic (-SH and -NH2) groups of proteins, peptides, and amino acids has been demonstrated and may account for deleterious effects of oxidized derivatives of chlorogenic acid (Stevenson et al., 1993).

2.2 Defense-Related Proteins

2.2.1 Maize Proteinase Inhibitor and Cysteine Proteinase

Protease inhibitors are synthesized and stored in seeds and tubers of plants. However, expression of some proteinase inhibitor genes is induced in response to mechanical wounding and insect damage. Local and systemic induction of expression of MPI, a maize protease inhibitor gene, was described by Cordero et al. (1994). MPI efficiently inhibits elastase and chymotrypsin-like activities from the larval midgut of *Spodoptera littoralis*. Hence, mode of action and expression profile suggests that MPI is a factor of maize insect resistance.

Representatives of tropical germplasm were found to exhibit resistance to Lepidoptera. In these lines, larval feeding led to the induction of a unique cysteine proteinase, Mir1-CP. Elevated Mir1-CP levels were found in the whorl in response to larval feeding (Pechan et al., 2000). Mir1-CP accumulates at the feeding site and is localized predominantly in the phloem of minor and intermediate veins (Lopez et al., 2007). Proteinase accumulation was correlated with a significant reduction in larval growth. A deleterious effect of Mir1-CP containing diet on the protective layer in the mid-gut of the larvae, the peritrophic matrix, has been detected. Consequently, nutrient utilization may be impaired and may account for the observed reduction in growth. Induction of gene expression by wounding is fast for

MPI and Mir1-CP, suggesting that signaling may be connected to the jasmonic acid pathway. In both cases induction levels are enhanced by damage caused by larval feeding, compared to mechanical wounding. An elicitor responsible for enhanced expression has not been identified.

2.2.2 Maize Ribosome-inactivating Proteins

Ribosome-inactivating proteins, (RIPs), share a site-specific RNA *N*-glycosidase activity and depurinate a universally conserved adenine residue of the large ribosomal RNA. RIPs impair susceptible ribosomes in translational elongation processes. RIPs are expressed by a wide variety of plants, an example of a highly toxic RIP is ricin from castor beans. Maize *Rip3:1* (old denomination *b-32*) is an abundant *Opaque-2*-regulated protein (>1 mg/g endosperm) associated with endosperm development. RIP3:1 requires proteolysis for activation. Proteolytic cleavage occurs during germination, but it has been shown that activation can also be performed by cysteine proteases found in several caterpillars and beetles. The association of increased insect susceptibility with RIP3:1 deficiency in *opaque-2* lines has led to suggestions that RIP can play a defensive role against insects. Effects of diets including activated RIP3:1 on mortality and weight gain of a wide range of caterpillars were assayed. Caterpillar susceptibility to the activated maize RIP appeared to be related to host adaptation, e. g. *Trichoplusia ni*, which does not feed on maize, was most severely affected (Dowd et al., 1998). The activated RIP protein is relatively stable to digestion by adversely affected caterpillar species. In a transgenic assay in tobacco, enhanced resistance to *Helicoverpa zea* was generated by constitutive expression of the activated maize RIP3:1 (Dowd et al., 2003). This finding provides support for the assumption that maize RIP plays a role in resistance to maize-feeding insects. Characteristics of RIP3:1, the developmentally regulated expression and synthesis of a non-toxic precursor, are reminiscent of the concept of phytoanticipine in the chemical defense strategy.

3 Genetics of Insect Resistance in Maize

3.1 QTL for Resistance to Tropical and Subtropical Maize Leaf Feeding Insects

Quantitative trait locus analysis has been used to examine the genetic basis of resistance in maize to the leaf feeding damage from tropical and subtropical insect pests of maize. The insects studied have included southwestern corn borer (SWCB), *Diatraea grandiosella*; sugarcane borer (SCB), *Diatraea saccharalis*; and the fall armyworm (FAW), *Spodoptera frugiperda*. SWCB and SCB were the focus of studies conducted at CIMMYT in collaboration with the laboratory of

A. E. Melchinger and SWCB and FAW by the USDA group at Mississippi State. The CIMMYT group used CML139 (Khairallah et al., 1998, Groh et al, 1998) and CML67 (Bohn et al., 1996, 1997; Groh et al., 1998) as the resistant parents to develop both F_2:F_3 (F_2-derived F_3 families) and RIL populations. A comparison of the QTL results across insects, populations and population structures (F_2:F_3 vs RIL) led to the following general conclusions. Within any particular experiment between 3 and 10 QTL of small to moderate effect were detected for a particular insect. There was often a good correspondence of QTLs between insects within a given experiment. Bohn et al. (1997) reported that in the CML131 × CML67 F_2:F_3 population, seven of 10 QTLs were shared for SWCB and SCB, likewise in the RIL population of the same cross all eight of the QTL for SCB were in common with the nine QTL for SWCB (Groh et al., 1998). In contrast to this high degree of pleiotrophy against the different insects, the commonality of QTL between population structures and resistant sources was much more limited. In comparing RIL to F_2:F_3 population structures for CML131 × CML67, four and five QTL were shared for SWCB and SCB (Bohn et al., 1997; Groh et al., 1998). For Ki3 × CML139 only one QTL for SWCB was shared between F_2:F_3 and RIL populations (Khairallah et al., 1998, Groh et al., 1998). In comparing resistant sources, for F_2:F_3 populations there were three shared QTL for resistance from CML139 versus CML67, located on chromosomes 5 (bins 5.06 and 5.07), and 9 (bin 9.05). In maize, the "bin" is a region of the genome of 15–20 cM bounded by specific markers (Gardiner et al., 1993). For the RIL populations only two QTL were in common, located on chromosomes 1 and 8. Groh et al. (1998) determined QTL for protein concentration and leaf toughness in the RIL populations. Although the genetic and phenotypic correlations of these traits with leaf feeding damage by SWCB and SCB were only low to moderate, a surprising number of common QTL positions were found suggesting that these traits may affect resistance. After re-evaluating all of these experiments by cross validation and independent sampling techniques, Bohn et al. (2001) expressed concern that the weaknesses in QTL detection across experiments and environment would limit potential to apply the results of these experiments by marker assisted selection (MAS). Willcox et al. (2002) conducted a pilot MAS experiment using CML67 as the resistance source and found that MAS was as effective as conventional selection (CS) and both selection procedures resulted in significant improvement in resistance over the susceptible parent, CML204, validating the prior QTL results.

Using the resistance sources Mp704 and Mp708, Brooks et al. (2005, 2007) mapped QTL for leaf feeding resistance to SWCB and FAW. These authors found that QTL on chromosomes 6, 7 and 9 were consistent for resistance to both insects and postulate that the candidate genes are the *mir* cysteine proteinase gene family (see Sect. 2.2) on chromosome 6 and the *Glossly15* gene on chromosome 9. *Glossly15* controls the adult to juvenile transition and adult leaves reduce FAW and SWCB growth and survival (Williams et al., 1998). Considering all of these studies on leaf feeding resistance, chromosomes 1, 5, 6, 7, 9 are commonly involved in resistance by what appears to be a number of different biological mechanisms.

3.2 QTL for Resistance to European Corn Borer

In the US Corn Belt there are two generations of ECB per season. Damage by the first generation ECB (1ECB) is predominately leaf-feeding on whorl stage plants. Damage by the second generation ECB (2ECB) is by sheath feeding and stalk and shank tunneling leading to stalk breakage and ear drop. In Europe there is one generation ECB that in Central Europe begins around anthesis and is similar to the second generation in the US. Schön et al. (1991) and Jampatong et al. (2002) mapped QTLs for resistance to the 1ECB using H99 and Mo47 as resistance sources. They reported common QTL in bins 1.06 and 6.02. Jampatong et al. (2002) also reported many QTL in the same chromosomal locations as QTL to the tropical leaf feeding insects suggesting a common genetic control. Jampatong et al. (2002) reported a large QTL in bin 4.01 consistent with the position of *bx* genes for DIMBOA synthesis. Resistance to 1ECB in temperate material is commonly related to DIMBOA levels in mid-whorl leaf tissue (Barry et al., 1994). Because of the established relationship of DIMBOA to 1ECB resistance, progress in selecting for 1ECB resistance has been relatively successful.

The issue of greater importance is selecting for resistance to the 2ECB stalk tunneling. This is reflected in the large number of QTL studies to determine the genetic basis of 2ECB resistance and to attempt to discover the biochemical basis of resistance. The sources of 2ECB resistance include the US temperate inbreds B52 (Cardinal et al., 2001, 2003; Cardinal and Lee, 2005) and De811 (Krakowsky et al., 2004, 2007), the European temperate line D06 (Bohn et al., 2000; Papst et al., 2004) and the temperate/tropical line Mo47 (Jampatong et al., 2002). Although each individual study generally identifies 3–10 QTL, some common themes have emerged. (1) Corresponding QTL positions are present among many of the studies, particularly on chromosomes 1, 5, 9. This result suggests that there may be some common biochemical basis for resistance among the different resistance sources. (2) QTL positions for tunnel length often overlap QTL for cell wall composition (CWC) traits. Cardinal and Lee (2005) reported that 10 of 13 QTL for tunnel resistance from B52 correspond with QTL for one or more of the CWC they studied. Krakowsky et al. (2007) found that nine of 10 QTL for tunneling resistance from DE811 co-localized with CWC QTL, although the direction of genetics effect often differed from that expected for resistance. Because of complex correlations among different CWC traits, it is difficult to identify specific biochemical constituents responsible for resistance. Papst et al. (2004) have proposed to use a candidate gene based association approach to attempt to clarify the resistance mechanisms controlled by the common QTL. (3) Many authors reflected on the overlap of QTL regions for tunneling resistance to ECB and leaf feeding resistance to tropical insects (Bohn et al., 2001; Cardinal et al., 2001; Jampatong et al., 2002) leading to speculation that leaf toughness traits for leaf feeding and CWC traits for 2ECB may share biochemical mechanisms. Flint-Garcia et al. (2003) demonstrated that MAS

using QTL for 2ECB can be as effective in selection as CS. Although the selection gains were relatively small, the results validate the QTL studies and suggest greater progress will be possible once the genes underlying the QTL are isolated and biochemical mechanisms elucidated.

3.3 Maysin and Corn Earworm Resistance

3.3.1 Genetic Regulation of Maysin Synthesis

The CEW is a major insect pest of maize in the Americas (Ortega et al., 1980). The adult CEW moths lay eggs on maize silks and the larvae access the kernels by feeding through the silk channel. Progress in understanding the chemical basis of native resistance to CEW began with the isolation of a C-glycosyl flavone, named maysin (Fig. 1), from silks of a CEW resistant Mexican landrace "Zapalote Chico" (Waiss et al., 1979, Ellinger et al., 1980). Snook et al. (1989, 1994) developed reversed phase high-performance liquid chromatographic procedures to quantify the amount of maysin and chlorogenic acid in maize silks. The biological effects of maysin, related flavones and chlorogenic acid on CEW growth and development were established through the development of artificial diet assays (Wiseman et al., 1992). The mechanism of antibiosis is an anti-nutritive effect (Summers and Felton, 1994).

The basic approach to understanding the genetic control of maysin synthesis was to develop F_2 or F_2:F_3 populations made by crossing maize lines known to differ in maysin levels and to conduct QTL analysis on the silk maysin concentrations or antibiosis as measured in the artificial diet bioassay. Traditionally, the interpretation of QTL studies has been limited by the lack of information on the metabolic pathways leading to most economic traits. Because the C-glycosyl flavones such as maysin are synthesized as a branch of the well characterized flavonoid pathway, a number of both regulatory and structural genes are known and can be applied as candidate loci in interpreting the QTL results. The synthesis of maysin and the genetic basis of antibiosis to the CEW became a model for understanding the genetic basis of agronomic traits.

The most common QTL for maysin and/or chlorogenic acid is in chromosome bin 1.03. This QTL is also often the largest QTL detected, commonly accounting for 40–60% of the variance in maysin concentration (Byrne et al., 1996, McMullen et al.,, 1998, Butron et al., 2001; Bushman et al., 2002). There is overwhelming evidence that the underlying gene is the *p* locus. The *p* locus encodes a Myb-homologous transcription factor (Grotewold et al., 1991). The *p* locus is complex and can contain two different but related *Myb* factors that were named *p1* and *p2* (Zhang et al., 2000). Zhang et al. (2000) demonstrated that while alleles of *p1* are expressed in pericarp, cob, tassel glumes and silks, *p2* is only expressed in glumes and silk. By studying deletions of *p1* and *p2*, Zhang et al. (2003) demonstrated that both *p1* and *p2* were capable of regulating maysin synthesis in maize silks.

Grotewold et al. (1998) and Bruce et al. (2000) demonstrated that expression of P1 protein in transgenic Black Mexican Sweet cells was sufficient to induce the synthesis of C-glycosyl flavones related to maysin and phenylpropanoids related to chlorogenic acid. A similar result was obtained with transformation of Black Mexican Sweet cells with the P2 protein expressed from a constitutive promoter (Zhang et al., 2003). Transformation of maize plants with a number of different *p1* alleles resulted in the accumulation of maysin in silks at levels sufficient for antibiotic effect (Cocciolone et al., 2005). The tissue specificity of expression of P1 in the transgenic plants was complex and indicative of epigenetic control of specificity. Szalma et al. (2005) conducted an examination of the relationship between *p* regulated phenotypes in maize pericarp, cob and silks, and the presence of *p1* and *p2* alleles in 76 maize lines. The phenotypes are given as a three letter designation as *p-XXX* with the first letter designating red or white pericarp (r or w), the second letter designating red or white cob glumes (r or w) and the third letter designating browning or non-browning silks (b or w). For 26 of 27 *p2* only lines the phenotype was *p-wwb*, that is white pericarps and cobs but presence of flavones in silks. Lines that were *p1* only or *p1* and *p2* could have a number of pericarp and cob phenotypes but all but one expressed the silk browning phenotype, that is, the lines were *p-wrb*, *p-rwb*, *or p-rrb*. Any population that is a contrast between functional and nonfunctional *p* alleles results in a major QTL at bin 1.03. Populations developed by crossing two functional, but distinct, *p* alleles can also result in a QTL in bin 1.03, but of much lower magnitude than the functional versus nonfunctional comparison (Meyer et al., 2007).

Chalcone synthase catalyzes the first committed step in flavonoid biosynthesis and is the branch point between the phenylpropanoid and flavonoid pathways. In maize, chalcone synthase is encoded by the duplicate loci *colorless2* (*c2*), located in bin 4.08 and *white pollen1* (*whp1*), located in bin 2.08. Therefore, the regulation and variation in expression of *c2* and *whp1* may be expected to be manifest as QTL for maysin synthesis. The chromosomal locations of *c2* and *whp1* have often been seen as containing QTL for maysin, related compounds and/or chlorogenic acid. Szalma et al. (2002) developed QTL populations incorporating mutant alleles of *c2* and *whp1* to test the role of each locus in maysin synthesis. Both the *c2* and *whp1* loci had an additive effect on maysin synthesis. Additional evidence that the QTLs in bins 2.08 and 4.08 are *whp1* and *c2* was obtained in gene expression studies by Meyer et al. (2007). In silks, both *whp1* and *c2* mRNA levels depended on *p1* genotype. A major regulatory point in maysin synthesis is P1 protein controlling the level of chalcone synthase, thereby controlling the amount of substrate entering the flavonoid pathway.

The maize anthocyaninless1 (*a1*) locus encodes NADPH dihydroflavonol reductase located in bin 3.09. The A1 protein is not involved in the synthesis of the flavones, but is required for anthocyanins and 3-deoxyanthocyanins which can also be produced in maize silks. It was therefore initially surprising that the *a1* locus was identified as a candidate QTL for maysin (Byrne et al., 1996). In maize silks the 3-deoxyanthocyanins are also regulated by the *p1* locus. McMullen et al. (2001) developed the (W23a1 × GT119)F_2 population to test if precursor shunting between

the 3-deoxyanthocyanin and flavone pathways in maize silks can be the basis of QLT effects for maysin. This population segregated for functional versus non-functional alleles at both *p1* and *a1*. The *p1* locus, *a1* locus and the *p1* × *a1* epistatic interaction were all highly significant QTL for both maysin and 3-deoxyanthocyanins. In the presence of at least one functional allele at *p1*, plants that were homozygous for the recessive *a1* allele accumulated twice the maysin as plants with functional *a1*. This result clearly demonstrated that controlling flux between alternative pathways can appear genetically as a major QTL effect. The basis of the epistatic interaction is also clear. Homozygous recessive *a1* can only induce enhanced maysin in the functional *p1* classes as both pathways must be active. Two sequence polymorphisms in the *a1* promoter were significant by association analysis for maysin suggesting that casual variation for the *a1* QTL may be transcriptional differences modulated by cis regulatory elements (Szalma et al., 2005). The QTL studies on maysin provide one of the best cases for the biological basis of epistatis for QTL effects for any plant system.

3.3.2 Maysin: How Much Is Possible? How Much Is Enough?

The level of maysin in maize silks in 2–3-day-old silks is generally in the range of 0.0–0.8% silk fresh weight (Snook et al., 1994), a very impressive accumulation for a secondary metabolite. Widstrom and Snook (2001) conducted recurrent selection in two populations, exotic populations of maize (EPM) and southern inbreds of maize (SIM), for high maysin levels. Selection was successful with an average 0.2% gain in maysin/per cycle of selection resulting in population means of ~1.5% fresh weight maysin. Meyer et al. (2007) investigated the genetic basis of the high maysin in inbreds derived from these selection populations and demonstrated that the major mechanism involved selection at alleles of *p*, *c2* and *whp1* resulting in increased flux into the flavone pathway. Meyer et al. (2007) also observed that a number of QTLs for high maysin differed between EPM and SIM. A new population was constructed by combining EPM and SIM. A single cycle of selection on this new population resulted in an increase population mean for maysin from 3.02% to 3.39% silk fresh weight (Meyer et al., 2007). There were individuals in the population with greater than 4% silk fresh weight maysin. This result shows how flexible cellular metabolism and physiology are in order to allow this high an accumulation of a "secondary" metabolite.

Based on these genetic studies we know the loci necessary to conduct MAS and develop lines with almost any desired level of silk maysin. Wiseman et al. (1992) demonstrated that 0.2% fresh weight silk maysin caused a 50% reduction in CEW larval weight gain in artificial diet feeding trials. So why has maysin not eliminated CEW as a pest on maize? The answer is that high maysin levels alone are not sufficient for field resistance (Rector et al., 2002). High maysin levels must be combined with tight and extended husk cover to force the CEW larvae to eat the silk rather than bypass the silk and move directly to feeding on kernels (Rector et al., 2002). This requirement for extensive husk cover removes maysin as a practical

control measure for CEW in the major feed grain producing areas of the US. Maize has been intentionally bred for short, open husks to permitted rapid drying of the grain in the field. The one market class of maize where maysin can play a viable role in host plant resistance to CEW is sweet corn, particularly the fresh market (Guo et al., 2001). Husk cover is desirable to protect the physical integrity of the kernels and can be used to force CEW and also FAW to ingest enough silk material to affect growth. Consumer preference for sweet corn is for a very clear pericarp and white cob. This preference would suggest that the best *p* alleles to use to engineer maysin into sweet corn would be *p-wwb* alleles (Szalma et al., 2005). Sweet corn lines with high maysin should gain consumer acceptance and help reduce the amount of chemical pesticides used to control CEW on fresh market sweet corn (Lynch et al., 1999).

4 Maize–Insect Tritrophic Interactions

Tritrophic interactions involving maize, herbivores, and enemies of the herbivores were first demonstrated two decades ago (Turlings et al., 1990). In these interactions, a blend of volatiles emitted from the herbivore-damaged plant attracts natural enemies of the herbivore. This phenomenon has been reported in more than 15 plant species (Dicke, 1999; Kessler and Baldwin, 2002; Meiners and Hilker, 2000). The attraction of herbivore enemies has been shown to benefit the plant by reducing subsequent herbivory and increasing reproductive fitness (Hoballah and Turlings, 1999; Van Loon et al., 2000; Kessler and Baldwin, 2001) although such advantages are not realized in all cases (Coleman et al., 1999). Therefore, these tritrophic interactions were also termed `indirect defense` of the plant (Dicke et al., 1990).

After damage of maize foliage by lepidopteran larvae, the plant releases a complex mixture of volatiles. The volatiles attract females of the parasitic braconid wasp *Cotesia marginiventris* (Hymenoptera), which oviposit on the larvae (Turlings et al., 1990). The parasitized lepidopteran larvae consume less plant material and will die upon emergence of the parasitoid, which can benefit the plant (Hoballah and Turlings, 1999; Hoballah et al., 2004). The composition of the lepidopteran-induced volatiles varies between different lines of maize and teosinte (Gouinguene et al., 2001; Degen et al., 2004) and is influenced strongly by abiotic factors like temperature, light intensity and nutritional status of the plant (Gouinguene and Turlings, 2002).

The maize volatile blend consists of indole, products of the lipoxygenase pathway, and a large number of mono- and sesquiterpenes (Turlings et al., 1990; Köllner et al., 2004). Attempts to identify the compounds which are crucial for the attraction of parasitic wasps have been hampered by the complexity of the blends and the difficulty of obtaining individual compounds with the correct chirality for bioassays (Turlings et al., 1991; D'Alessandro and Turlings, 2005, 2006). Fortunately, identification of genes involved in the biosynthesis of these volatiles has provided

molecular tools to demonstrate which of the compounds are attractive to the parasitic wasp. The major sesquiterpene volatiles of herbivore-induced maize are produced by the terpene synthase TPS10 which is strongly expressed after herbivory by Lepidoptera. TPS10 forms (*E*)-β-farnesene, (*E*)-α-bergamotene, and other herbivory-induced sesquiterpene hydrocarbons from the substrate farnesyl diphosphate (Schnee et al., 2006). Overexpression of TPS10 in *Arabidopsis thaliana* resulted in plants emitting high quantities of TPS10 sesquiterpene products identical to those released by maize. Using these transgenic Arabidopsis plants as odor sources in olfactometric assays showed that females of the parasitoid *Cotesia marginiventris* learn to exploit the TPS10 sesquiterpenes to locate their lepidopteran hosts after prior exposure to these volatiles in association with the host (Schnee et al., 2006). This gene-based dissection of the herbivore-induced volatile blend demonstrates that a single gene such as *tps10* can be sufficient to mediate the indirect defense of maize against herbivore attack. Furthermore, associative learning can also adapt parasitoids to alterations of the herbivore-induced volatile blend by plant species, age and tissue of the plant, and abiotic conditions (Takabayashi et al., 1994; De Moraes et al., 1998; Schmelz et al., 2003; Van den Boom et al., 2004). However, females of *Cotesia marginiventris* are also attracted to the full blend of maize volatiles without prior association, indicating that the blend contains additional attractive compounds that elicit an innate response (Hoballah and Turlings, 2005). Interestingly, the emission of volatiles after herbivore damage is not always beneficial for the maize plant since larvae of FAW, another lepidopteran species, use these volatiles to locate their food plants (Carroll et al., 2006).

Terpene-mediated interactions were not only observed in response to damage of the leaves but also in response to root-feeding herbivores. Larvae of the beetle *Diabrotica virgifera virgifera* (Western corn rootworm) are an important pest of maize. In response to feeding by the larvae, maize roots release a signal that strongly attracts the entomopathogenic nematode *Heterorhabditis megidis* (Boff et al., 2001; Van Tol et al., 2001). The signal released by the maize roots was identified as (*E*)-β-caryophyllene, a sesquiterpene olefin. Most North American maize lines do not release (*E*)-β-caryophyllene from the roots, whereas many European lines and the wild maize ancestor, teosinte, do so in response to *D. v. virgifera* attack. Field experiments showed a fivefold higher nematode infection rate of *D. v. virgifera* larvae on a maize variety that produces the signal than on a variety that does not. Spiking the soil near the latter variety with authentic (*E*)-β-caryophyllene decreased the emergence of adult *D. v. virgifera* to less than half (Rasmann et al., 2005).

In an agricultural setting, the value of indirect defense has been shown by the co-cultivation of maize with an African grass (*Melinis minutiflora*) that releases abundant volatile compounds (Khan et al., 1997, 2000). The proximity of this grass led to a significant reduction in damage to maize plants by lepidopteran larvae due to an increased parasitism by braconid wasps. These results suggest that the manipulation of volatile emission in maize may be a valuable strategy to attract herbivore enemies and thus minimize pest problems in an environmentally safe manner. This strategy might be aided by engineering of maize plants that emit strong, readily detectable volatile signals that match the preferences of particular enemy species

(Degenhardt et al., 2003; Turlings and Ton, 2006). The development of such plants is now feasible due to the elucidation of the pathways responsible for the biosynthesis of volatile compounds. The effectiveness of these tritrophic interactions is most likely in synergy with the direct defenses of the plant (e.g., toxins or feeding deterrents) which extend the time that herbivores remain vulnerable to attack from foraging enemies. Further studies of the interactions between maize, their herbivores and the enemies of their herbivores should provide more clues to facilitate the application of indirect defenses in the cultivation of maize.

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