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INDUCTION AND METABOLIC ENGINEERING OF FLAVONOID DEFENSE COMPOUNDS IN SORGHUM AND MAIZE

A Thesis in

Plant Physiology

by

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ABSTRACT

Sorghum is closely related to maize but has a better ability to withstand biotic and abiotic stress. To gain insights into the possible reasons underlying such difference, a comparative characterization of genes and traits has been conducted. We have investigated the flavonoid pathway in these two species. This research indicated that sorghum and maize differ substantially in their ability to synthesize flavonoid compounds. For example, sorghum plants respond to anthracnose and leaf blight fungi by synthesis of flavonoid antifungal compounds (phytoalexins). However, maize plants do not produce simillar compounds in response to fungal attack. Therefore, we used sorghum as a model system to understand the biosynthetic routes of the flavonoid phytoalexins and attempted to engineer the production of these compounds in maize.

Sorghum phytoalexins belong to the 3-deoxyanthocyanidin class which includes luteolinidin, apigeninidin and their derivatives. These compounds are produced in response to fungal attack and appear as red-brown pigments at the primary infection sites. Their antifungal activity against many fungi has been demonstrated; however, the lack of well defined phytoalexin deficient mutants has hampered the understanding of the mechanisms underlying their biosynthesis. These compounds have chemical structures similar to that of flavan-4-ols, the precursor of phlobaphene pigments that appear in sorghum floral tissues and mature leaves, suggesting that these two flavonoid classes may be synthesized via a common or an overlapping pathway. The biosynthesis of flavan-4-ols is under the control of an R2R3 myb transcription factor encoded by *yellow seed1* (*y1*).

To gain a better understanding of the possible role of *y1* in controlling sorghum phytoalexins, we used a transposon-based approach and developed near-isogenic sorghum lines that differ in the functionality of *y1*. Molecular and phenotypic analyses of one of these lines (*y1-ww*) revealed the presence of an internal deletion in *y1* and such deletion rendered *y1* non-functional. The gene expression analysis of the fungal inoculated isogenic lines indicated that the expression of the phytoalexin biosynthetic genes is *y1* dependent. Comparison of the phytoalexin biosynthetic ability of these lines demonstrated that *y1* mutant is phytoalexin deficient. The *y1* activity was also positively correlated with the resistance against anthracnose disease. These results provide direct genetic evidence that *y1* is necessary for the biosynthesis of sorghum 3-deoxyanthocyanidins and resistance against anthracnose disease.

Like sorghum, maize accumulates flavan-4-ols in the floral tissues but not in leaves. The biosynthesis of these compounds in maize is under the control of *pericarap color1* (*p1*). *p1* and *y1* are orthologues with a high degree of similarity in their coding sequences but very low similarity in the regulatory regions . These two genes have almost the same pattern of expression except that *y1* is significantly expressed in leaf and is induced by fungal infection. Therefore, we developed transgenic maize lines expressing either an *Y1::GUS* fusion or the intact *y1* gene driven by its own promoter. These plants were used to test the hypothesis that the expression of *y1* in leaves and its induction by the fungus is a property of the *y1* promoter. Analysis of the *Y1::GUS* plants revealed that *y1* promoter has constitutive low expression in maize floral and vegetative tissues and is responsive to fungal infection. Interestingly, *y1* induced phlobaphene pigmentation in

maize floral tissues and these phenotypes were stably inherited through generations. In addition, *y1* successfully drives the flavonoid pathway in maize leaves towards the biosynthesis of flavan-4-ols, and induces the biosynthesis of indigenous as well as novel flavonoid and phenylpropanoid compounds in maize leaves. The accumulation of these defense-related compounds in transgenic maize leaves resulted in an enhanced resistance against corn southern leaf blight. These results suggest that possible evolutionary modifications in *y1* and *p1* promoters might be responsible for the differential flavonoid profiles of sorghum and maize in response to fungal attack.

The biosynthesis of sorghum phytoalexins occurs rapidly after fungal attack and involves activation of *y1* and its target genes. The signaling cascades upstream of the biosynthesis of these compounds are unknown. The dissection of the *y1* promoter revealed the presence of *cis* recognition sequences that have been shown to be targets of signaling compounds such as JA, ABA, and WRKY proteins. Simultaneous phytohormonal analysis during 3-deoxyanthocyanidins biosynthesis indicated that sorghum phytoalexins are associated with early induction of JA and IAA, and either no change or reduction of ABA respectively. A hypothetical model for signal transduction during sorghum phytoalexin biosynthesis is proposed

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Chapter 1

General introduction

1.1 General plant defense mechanisms

Plants are immobile and are continuously threatened by wide range of pathogenic and nonpathogenic microbes in their natural and agricultural systems. To protect themselves against these microbes and to retain their fitness, they have evolved different defense mechanisms. Many plants have mechanical barriers even before the potential pathogens arrive. These barriers are called passive defense mechanisms and include dense leaf hairs, thick cuticle and cell wall to prevent pathogen from contacting or penetrating host tissues. Another passive mechanism is having preformed chemicals called phytoanticipins. These compounds may be effective against weak pathogens (Van Etten et al., 1994; KotrteKamp and Zybrian, 1999; Chassot and Métraux, 2005).

Failure of the passive defense mechanisms in stopping pathogen attack leads to penetration of the host tissues and this initiates the active defense mechanisms within the plant. The consequences of pathogen ingress are greatly determined by how rapidly the host recognizes the presence of the invading pathogen and how efficient the induced defense mechanisms are. Recognition of the pathogen by host triggers one or more active defense mechanisms to restrict pathogen development within plant tissues. Generally,

these mechanisms involve redirection of plant metabolism toward production of active oxygen species, lignifications of cell wall, induction of pathogenesis related proteins and production of secondary metabolites or phytoalexins (Lamb et al., 1989; Dixon and Lamb, 1990; Nicholson and Hammerschmidt, 1992; Hammond-Kosack and Jones, 1996; Dixon, 2001).

1.2 Secondary metabolites are necessary for plant defense

Although all plants have common end products of primary metabolism, they differ considerably in their profiles of secondary metabolites. Both primary and secondary metabolic pathways share common precursors (Herms and Mattson, 1992). One essential link between plant primary and secondary metabolism is governed by phenylalanine ammonia lyase (PAL). It converts the phenylalanine, a shikimic acid pathway product, to cinnamic acid, a phenylpropanoid intermediate (Hahlbrock and Scheel, 1989). The evident diversity in the profiles of secondary metabolites among plants belonging to different taxonomical groups has been suggested to reflect the evolution history of these plant taxa (Gottlieb, 1989, 1990).

In spit of the fact that many secondary metabolites have been proven to play a primary role in many physiological processes, special attention has been given to their role in plant defense. The phytoalexin concept was introduced by Muller and Borger in 1940 to describe chemicals produced by potato tissues in response to *phytophthora* infection (Purkayastha, 1994). Currently, phytoalexins are defined as low molecular weight compounds produced and accumulated *de novo* in plants in response to infection

or stress (Purkayastha, 1994; Van Etten et al., 1994; Hammerschmidt, 1999). For any compound to act as phyhytoalexins, this compound should be produced at the time of attempted pathogen attack (right time), in host cells under challenge (right place), and in concentration high enough to kill the pathogen (Cooper et al., 1996).

The phytoalexin activity of a wide variety of secondary metabolites belonging to different chemical classes has been discovered in different plant families. Specific examples include isoflavonoids and pterocarpans in legumes (Paxton, 1995), sulphur containing indole phytoalexins found in cruciferous family (Hammerschmidt, 1999), sesquiterpenoid phytoalexins produced in solanaceous plants (Brooks and Watson, 1991), and coumarin phytoalexins in umbelliferous plants (Knogge et al., 1987). In rice and sorghum, flavonoid compounds have been shown to act as phytoalexins against *Magnaporthe grisea* and *Colletotrichum sublineolum* species, respectively (Snyder and Nicholson, 1990; Kodama et al., 1992).

1.3 Flavonoids, a major class of secondary metabolites.

Flavonoids are polyphenolic secondary metabolites with a C_{15} carbon skeleton. These compounds are ubiquitous in *planta* and form one of the most abundant classes of secondary metabolites. Accumulation of these compounds leads to pigmentation of plant tissues including flowers, fruit, seeds, and plant body. Flavonoids include many subclasses such as chalcones, dihydrochalcones, flavanones, flavanols, flavones, flavonols, anthocyanin, anthocyanidins, 3-deoxyanthocyanidins, deoxyflavonoids (Harborne and Williams, 2000). Anthocyanins represent the most obvious flavonoids

while 3-deoxyanthocyanidins are uncommon (Winefield et al., 2005). Although flavonoids are secondary metabolites, they play a primary role in both plant and animal life.

1.4 Flavonoids have many biological functions

1.4.1 Flavonoids have potential health benefits

Plant derived flavonoids are essential components of human diets. Because of their low molecular weight and the ease of their absorption by human body, these compounds have been considered among the safest non-immunogenic drugs. These compounds are effectively interacting with human metabolism and their activities against many human disorders have been reported. Specific examples for their anticancer activities include: quercetin, kaempferol and many other flavonoids against leukemia, anthocyanidins against stomach and colon cancer. Amazingly, deguelin, a flavonoid from *Mundulea serica*, was one component of a mixture that inhibits growth of premalignant and malignant lung cancer cells. In addition, anthocyanin rich extract prepared from grape, bilberry and chokeberry suppresses the progression of colon carcinoma cell cycle (Lee et al., 2007). Recently the rare flavonoid class, 3-deoxyanthocyanidins from sorghum, has been demonstrated to reduce proliferation of human blood and liver cancer cells (Shih et al., 2007). Apart from their role as anticancer compounds, flavonoids are

also effective against cardiovascular disorders, diabetes, and neural diseases (Lee et al., 2007).

1.4.2 Flavonoids are important for plant reproduction

Flavonoids, particularly flavanol, facilitate plant reproduction by attracting pollinators, seed disseminators, promoting pollen germination and tube growth (Koes et al., 1994; Taylor and Miller, 2002; Escribano-Bailon et al., 2004). Flavonoids are also necessary for pollen fertility in many plant species. For example, in maize and petunia, the lack of flavonols, due to mutation in chalcone synthase, led to pollen sterility (Coe et al., 1981; Napoli et al., 1999). Interestingly, the pollen sterile petunia mutant phenotype was complemented either by exogenous application of flavanol or by transformation of the deficient mutant with *chs* transgene (Mo et al., 1992; Napoli et al., 1999).

1.4.3 Flavonoids are involved in plant-symbionts interactions

Flavonoids have been implicated in regulation of plant-*Rhizobium* symbiosis in legume roots. These compounds act, first, as chemo-attractants for the *Rhizobium* bacteria and then as inducers of their nod genes (Eckardit, 2006). The induction of *Rhizobium meliloti* nod gene by Alfalfa derived luteolin, 3', 4', 5, 7-tetrahydroxyflavone; 4', 7-dihydroxyflavone, 4', 7-dihydroxyflavanone, 4, 4'-dihydroxy-2'-merthoxychalcone has been reported (Peters et al., 1986; Maxwell et al., 1989). Interestingly, root extracts from

nitrogen deficient Alfalfa plants accumulated high level of the non glycosylated 7,4'-dihydroxyflavone whose induction effect on *Rhizobium meliloti* nod gene was also reported (Coronado et al., 1995). It was suggested that the flavonoid aglycone diffuses into the rhizobial bacteria to induce nod genes which encode nod factors (bacterial lipochitin-oligosaccharides) that initiate nodule formation (Kobayashi et al., 2004). Recently, it has been suggested that bacterial nod factors are perceived by a plant receptor and this recognition induces a transient inhibition of auxin transport to favor nodule formation (Eckardit, 2006; Wasson et al., 2006). These results suggested that under nutrient stress, plants direct their secondary metabolism to produce flavonoids that enhance the symbiotic relations with bacteria to secure their nutrient needs.

1.4.4 Flavonoids play a role in adaptation of plants to nutrient stress

The nutritional status of many plant systems significantly influences their flavonoid content. For example, growing Arabidopsis and Tomato seedlings on nitrogen and phosphorus deficient MS media induces highly significant increase in the total flavonoid content. The increment in flavonoid content was inversely proportional to nitrogen and phosphorus availability (Stewart et al., 2001). In Arabidopsis plants over-expressing *PAP1*, a positive regulator of the flavonoid pathway, flavonoids content was significantly induced after growing these plants on nitrogen deficient soil, hydroponics, or on agar plates (Lea et al., 2007). Here, it might be important to mention that nutrient stress results in disruption of photosynthetic membranes and decrease in chlorophyll content which eventually leads to increased sensitivity to high light intensities. Apart

from the induction of flavonoids in response to nutrient deficiency, a significant induction of synthesis of these compounds has also been reported in response to high level of toxic metals like aluminum. For example, exogenous application of Si to Aluminum-resistant maize cultivars induced increase in catechin and quercetin content up to 15 folds in root exudation more than in untreated plants (Kidd et al., 2001). These findings shed light on the possible role of flavonoids in protecting plants against nutrient stress.

The potential roles that flavonoids can have under nutrient stress include securing alternative resources for the deficient element and protecting internal plant tissues from consequences of such stress. For example, flavonoid biosynthesis is coordinated with increase in PAL activity which leads to liberation of more nitrogen from phenylalanine for amino acids and protein metabolism (Margna, 1977). In addition, flavonoids can minimize the stress-induced sensitivity to high light intensities (Guidi et al., 1998). Flavonoids are also proposed to enable plants to adapt to soil rich in toxic elements (Barcelo and Poschenrieder, 2002). Because of their ability to bind Aluminum ions, flavonoids have been suggested to reduce both external and internal toxicity of Al ions by preventing their binding to cell wall and plasma membrane of root cells. Furthermore, flavonoids play a role in controlling the redox state of cells under stress by scavenging the nutrient stress-induced ROS (Babu et al., 2003; Kochian et al., 2004; Shin et al., 2005).

1.4.5 Flavonoids take part in plant growth and development

Flavonoids have been demonstrated to play a role in controlling general aspects of plant development via their interaction with plant growth regulators like auxin. Inhibition of auxin transport by flavonoid has been reported (Jacobs and Rubery, 1988; Murphy et al., 2000). Arbidopsis *chs* mutants exhibited increased level of auxin transport and showed many developmental aberrations including secondary root growth, diminished plant height and increased number of inflorescence. These mutants also exhibited delayed gravitropism and can be complemented by supplementing naringenin (Taylor and Grotewold, 2005). Also, distorted leaf morphology, hindered root growth, retardation of cell division and differentiation was reported in flavonoids-deficient alfalfa plants (Woo et al., 2002). The flavonoid-induced inhibition of auxin transport might attribute to the structural similarity of flavonoid to naphthyphthalamic acid (NPA), an auxin transport inhibitor (Jacobs and Rubery, 1988).

1.4.6 Flavonoids are involved in modulation of enzyme activities

Because of their antioxidant as well as redox nature, flavonoids have been suggested to play a role in modulation of enzyme activities. Particular attention has been given to enzymes that are redox sensitive and vulnerable to reactive oxygen species (Moini et al., 2002). In addition, it is established that proanthocyanidins are very efficient in binding proline rich proteins (Hagerman and Butler, 1981). The inhibitory effect of different flavonoids is reported for cow's milk Xanthine oxidase (Iio et al., 1985),

glutathione reductase (Elliott et al., 1992), photosynthetic enzymes, NADP-malic dehydrogenase, phosphoenol pyruvate carboxylase, (Pairoba et al., 1996), rabbit and soybean 15-lipoxygenases (David et al., 2003), glycogen phosphorylase (Jakobs et al., 2006), rat ά-glucosidase and ά-amylase (Tadera et al., 2006).

Interestingly, it has been suggested that the redox state of a cell controls the activity of R2R3 MYB transcription factors, including those regulating flavonoid biosynthetic pathway (Heine et al., 2004). Interestingly, the MYB domain of P1, an R2R3 myb protein that regulates a branch of the flavonoid pathway, has been demonstrated to bind DNA *in vitro* only when reduced, a condition that can be fulfilled by DTT *in vitro* (Heine et al., 2004). Because flavonoids are antioxidant, under stress they can contribute to controlling the redox state of cells by scavenging the reactive oxygen species and so they can contribute to regulation of MYB proteins (Heine et al., 2004).

1.4.7 Flavonoids have many ecological functions

The potential contribution of flavonoids to plant-environment, -plant, -insect and plant–pathogen interactions have been extensively studied in many plant systems. The defense related flavonoids might be pre-made or induced compounds. The pre-made flavonoids are synthesized during the normal plant development and are usually associated with nonhost resistance while the induced flavonoids accumulate in response to adverse types of stress (Nicholson and Hammerschmidt, 1992; Treutter, 2005).

1.4.7.1 Flavonoids protect plants against UV radiation

Flavonoids play a crucial role in protecting plants under stress. Because of their high antioxidant activity, their accumulation in stressed tissues has been considered as part of a general stress response. Since flavonoids accumulate mainly in the epidermal tissue and have strong UV absorbing capabilities, they have been suggested to form a filter to protect DNA, proteins and photosystem II reaction center from the deleterious effect of UV radiation (Woo et al., 2005). Interestingly, *Arabidopsis thaliana* plants carrying mutations in *chs* and *chi* showed retarded growth under UV illumination compared to wild type plants (Li et al., 1993). On the other hand, *Arabidopsis thaliana* plants that have high endogenous levels of flavonoid and phenolic compounds were resistant to high levels of UV-B (Bieza and Lois, 2001).

1.4.7.2 Flavonoids contribute to plant-plant interactions

Flavonoids play an essential role in allelopathy, a case in which one plant releases phytotoxic chemicals to restrict growth of other neighboring plants. For example, the invasive nature of *Centaurea maculosa* was attributed to its ability to release the flavanol (−)-catechin in the rhizosphere. This compound has been shown to trigger cell death mechanisms in the neighboring plants (Bais et al., 2003). Many other flavonoid compounds, including chlocones, flavanones, dihydroflavonols, have been reported to exhibit inhibitory effect on germination, growth and development of other plants (reviewed in Iwashina, 2003).

1.4.7.3 Flavonoids contribute to plant resistance against insects and fungal infections

Flavonoids have been recognized as feeding deterrents and mortality inducer for many insect because of their taste, ability to reduce food digestibility and action as toxins. The deterrence and insecticidal activities of flavonoids has been demonstrated against several insects including *Spodoptera* species (flavanones), *Acyrthosiphum pisum* (dihydrochalcones), *Eurema hecabe mandarina* (flavans), *Heliothis zea* (corn earworm, C-glycosyl flavone), *Pectinophora gossypiella (*flavonols, quercetin and its derivatives) *Schizaphis graminum* (procyanidins) and *Coptotermes formosanus* (apigenin and quercetin) and so on (Waiss et al., 1979; Widstrom and Snook, 2001; Boue and Raina, 2003; Iwashina, 2003). Apart from their importance in plant resistance against insects, flavonoids play an essential role in protection of plants against fungal diseases, and this will be the scope of the rest of this review.

1.5 Flavonoid phytoalexins

Many flavonoids have been reported to accumulate at the site of pathogen penetration and prevent further development of pathogen within plant tissues. Flavonoid phytoalexins have been discovered in gymnosperms, monocotyledones and dicotyldons (Harborne, 1999; Iwashina, 2003). Whether there is a systematic significance of these compounds in *planta* is still far from clear.

Flavonoid phytoalexins have been isolated from different plant species and include Aurone, chalcones, dihydroflavonols, flavans, flavan 3-ols, Flavan 4-ols,

flavanones, flavones, flavanols, C-glycosylflavones, and isoflavonoids. It is noteworthy that one of the unique properties of monocots is the ability of certain species to produce intensive amounts of a rare class of flavonoids called 3-deoxyanthocyanidins. At least sorghum and sugarcane have been reported to produce such compounds in response to fungal infection (Nicholson et al., 1987; Viswanathan et al., 1996).

The possible mechanisms for inhibiting the pathogen development by flavonoid phytoalexins include: disruption of pathogen plasma membrane, cross-linking the microbial enzymes, suppression of microbial cell wall degrading enzymes like cellulases, pectinases and xylanases, and chelation of microbial enzyme activators such as metal ions (Skadhauge et al., 1997; Nielsen et al., 2004)

1.6 The flavonoid pathway

The flavonoid biosynthetic pathway is one of the best understood pathways in plant secondary metabolism. It has been intensively studied in many plant species and it is established that the initial precursors necessary for flavonoid biosyhthesis are derived from shikimic acid and phenylpropoanoid pathways. These three pathways function in close coordination to secure the efficient flow of carbon from primary metabolism to phenylpropanoid and flavonoid pathways for the production of multifunctional secondary metabolites within normal cells or in cells under stress (Douglas, 1996).

The shikimic acid pathway begins with the reaction of phosphoenol pyruvate (a product of glycolysis) with erythrose-4-phosphate (a product of the pentose phosphate pathway) in a reaction catalyzed by 3-deoxy-D-arabino-heptulosonate-7-phosphate

synthase (DAHP synthase). This pathway generates, via chorismate, three essential amino acids: phenylalanine, tryptophan, and tyrosine. Phenylalanine is the entry point of phenylpropanoid pathway. In this pathway, phenylalanin ammonia lyase (PAL) deaminates phenylalanine to cinnamic acid (Hahlbrock and Scheel, 1989), which undergoes aromatic hydroxylation by C4H to form p-coumaric acid. Then 4CL catalyzes the activation of p-coumaric acid to form p-coumaroyl CoA. These compounds could be shunted to either simple compounds such as chlorogenic acid, flavonoids or to complex compounds such as lignin (Fig 1).

Flavonoid biosynthesis starts with activation of chalcone synthase (CHS) which catalyzes the stepwise condensation of one molecule of coumaryl CoA and three molecules of malonyl-CoA to form chalcone. Chalcone then undergoes stereospecific isomeration by CHI to form naringenin, the common precursor of many flavonoid pigments including anthocyanins, 3-hydroxyflavonoids, 3-deoxyflavonoids, and 3-deoxyanthocyanidins. The fate of naringenin is determined by the genetic constituents of the plant and the environmental conditions as well (Fig 1).

During the synthesis of anthocyanins and 3-hydroxyanthocyanidins, naringenin is hydroxylated at the 3-position by the activity of flavanone 3-hydroxylase (F3H) to dihydroflavanol which is then reduced by dihydroflavanol reductase (DFR) to provide the intermediates of common anthocyanin. Bypassing the activity of F3H gives rise to formation of flavan 4-ols (apiferol and luteoferol) via naringenin and/or eriodictyol. In fact, incubation of C^{14} labeled narigenin and eriodictyol with enzyme preparation from either maize silk or Sinningia cardinalis led to formation of apiferol and luteoferol, respectively (Stich and Forkmann, 1988; Halbwirth et al., 2003). Flavan 4-ols have been

suggested to polymerize to form the red pigment phlobaphene (Styles and Ceska, 1989; Grotewold et al., 1994).

Flavan 4-ols were also suggested as direct precursors of 3-deoxyanthocyanidins (Stich and Forkmann, 1988; Halbwirth et al., 2003; Winefield et al., 2005). This was based on the structural similarities and the concomitant presence of flavan 4-ols and 3-deoxyanthocyanindins in sorghum (Bate-Smith, 1969; Watterson and Butler, 1983). Thus for synthesis of 3-deoxyanthocyanidins, plants have to suppress the activity of F3H and activate the reduction activity of naringenin. In fact, reduction of anthocyanin biosynthesis via suppression of flavanone 3-hydroxylase, dihydroflavonol 4-reductase, and anthocyanidin synthase during biosynthesis of sorghum 3-deoxyanthocyanidins has been reported (Lo and Nicholson, 1998). In addition, during the formation of flavan 4-ols in maize pericarp, the activities of CHS, CHI, and DFR but not F3H has been demonstrated to be controlled by an R2R3 myb transcription factor encoded by *pericarp color1* (*p1*) (Grotewold et al., 1994).

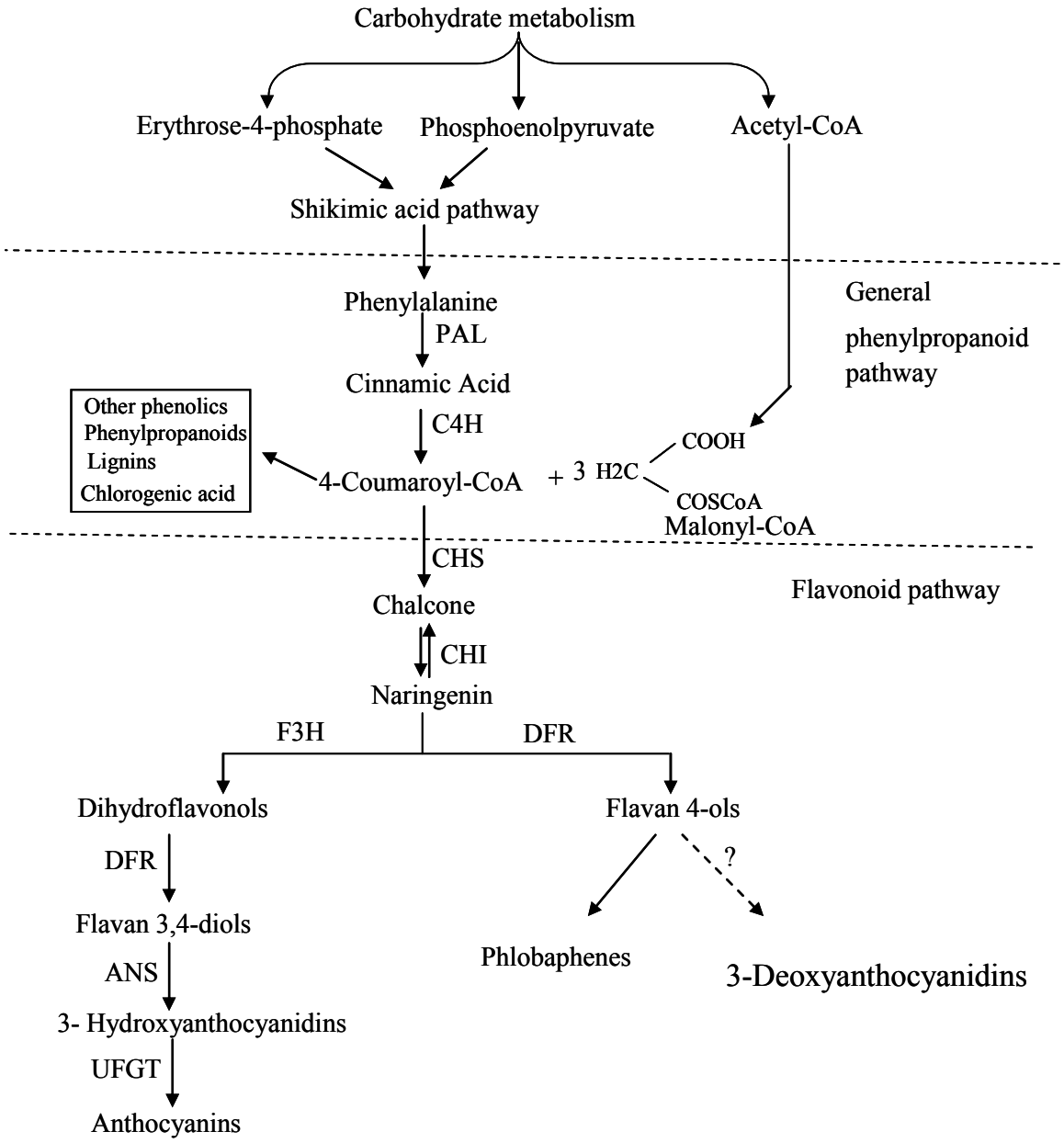

Figure 1 General phenylpropanoid and flavonoid pathways. Enzymes are: PAL, phenylalanin ammonia lyase; C4H, cinnamate 4 hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavaonol reductase; ANS, anthocyanidin synthase; UFGT, UDP glucose flavonoid glucosyl-transferase.

1.7 Genetics of flavonoids

Since Mendel's studies, genetics of flavonoid pigments has been an important constituent of classical and molecular genetics. The massive accumulation of biochemical and genetic data about flavonoid genes has made them one of the most preferred research topics. Intensive effort has been put to characterize the flavonoid pathway at the molecular level (Growewold, 2005).

The flavonoid pathway is driven by two main groups of genes, the structural and the regulatory genes. Structural genes include those that control individual steps in the pathway and those that shape the final chemical structure of these compounds. The regulatory genes include genes that activate, repress a part or the whole pathway and those controlling the level of flavonoid accumulation. To date, genes that control flavonoid biosynthesis have been best studied in maize, petunia and antirrhinum (Forkmann, 1994; Growewold, 2005).

1.8 Flavonoid biosynthesis in sorghum and maize

Sorghum and maize have been predicted to be diverged from the same ancestor about 20 million years ago (Doebly et al., 1990; Bennetzen and Freeling, 1997). Sorghum genome (750 million base pair) is three times smaller than that of maize (2500 MB) (Arumuganathan and Earle, 1991), and both genomes show high degrees of synteny and sequence homology in many genes (Melake-Berhan et al., 1993; Devos and Gale, 2000). For example, *sh2* and *a1* were found to have the same arrangement in the two genomes

but they are 140 kb apart in maize while only 19 kb in sorghum (Chen et al., 1997). Because of the co-linearity between these two species, it has been suggested that understanding metabolic pathways in one species could be of great help to understand the metabolism of the other.

1.8.1 Flavonoid pathway in maize

Maize plants synthesize two essential classes of flavonoids including 3-hydroxyflavonoids (anthocyanins) and 3-deoxyflavonoids (phlobaphenes). Although these two classes are derived from a common flavanone precursor, naringenin, they show differential pattern of localization within maize tissues. Anthocyanins can be present in most maize organs while pholobaphenes are floral tissue specific (Styles and Ceska, 1975; Dooner et al., 1991; Van der Meer et al., 1993; Grotewold et al., 1994).

The synthesis of these two flavonoid classes is under the control of independent classes of transcription factors. The biosynthesis of anthocyanin is regulated by two sets of transcription factors including those with MYB domain (*C1* and *Pl*) and those with bHLH domain (*R* and *B*) (Ludwig et al., 1990; Dooner et al., 1991; Van der Meer et al., 1993). These regulatory proteins interact together to activate their downstream structural genes in the anthocyanin biosynthetic pathway. On the other hand, phlobaphene biosynthesis is regulated by only one R2R3 MYB protein P1, a product of *pericarp color1* (*p1*) (Styles and Ceska, 1977; 1989). *p1* has been cloned (Grotewold et al., 1994) and demonstrated to activate the flavonoid downstream genes by binding to the

$CC^{T}/_{A}ACC$ in the promoter regions of these genes. This recognition has been demonstrated at the molecular level for *A1* promoter (Grotewold et al., 1994).

A number of different *p1* alleles have been characterized. These alleles have been named based on the pattern of their pericarp and glume pigmentation. For example, *P1-rr*, describes red pericarp and red cob glume, *P1-wr*; white pericarp and white glume; *P1-rw*; red pericarp and white glume, *p1-ww* for white pericarp and white glume, and *P1-vv* stands for variegated pericarp and variegated cob glumes (Fig. 2) (Anderson, 1924). It is worth mentioning here that the variegated phenotype in maize was due to the insertion of AC transposone in *p1* locus (Athma et al., 1992).

1.8.2 Flavonoid pathway in sorghum

Sorghum plants synthesize anthocyanin, 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and 3-deoxyflavonoids (phlobaphene) in response to environmental stimuli or during normal plant development (Kambal and Bate-Smith, 1976; Snyder and Nicholson, 1990; Zanta et al., 1994; Lo and Nicholson, 1998). The biosynthesis of anthocynin is controlled by transcription factors *C1*/*Pl*1 and *R*/*B.* For example, following illumination, sorghum mesocotyls accumulated *C1* transcripts as early as 3 hours post treatment. These plants accumulated anthocyanin compounds including mono and dimalonyl derivatives of cyanidin-3-O- glucoside. Interestingly, none of the *C1* transcripts were detected in 3-deoxyanthocyanidins producing sorghum in response to infection with *C. heterostrophus*. These results indicated that 3-

deoxyanthocyanidins biosynthesis seems to be under transcriptional control different from that of anthocyanin (Hipskind et al., 1996).

Phlobaphene accumulation is obvious in the floral tissues and in the mature leaf as well. In fact, there is a striking similarity between the pericarp pigmentation phenotypes between sorghum and maize. For example, sorghum grain pigmentation patterns including red, white, and candystripe phenotypes have their comparable phenotypes in maize. This suggested that synthesis of phlobaphene pigment in sorghum might be under the control of *p1*-ortholog. Indeed, the unique similarity between variegated maize and candystripe sorghum pericarp led to the cloning of a MYB transcription factor *yellow seed1* (*y1*) (Chopra et al., 1999; Boddu et al., 2005). It has been demonstrated that *y1* encodes an R2R3 myb transcription factor highly similar to maize *p1* and controls phlobaphene pigmentation in sorghum floral tissue and in mature leaves (Kambal and Bate-Smith, 1976; Zanta et al., 1994; Chopra et al., 1999; Boddu et al., 2005; Carvalho et al., 2005; Boddu et al., 2006).

y1 alleles have been named using the same nomenclature system as that used for *p1* alleles (Chopra et al., 1999; 2002; Boddu et al., 2004; 2005; 2006). For example, *Y1-rr* stands for red pericarp and red glumes, *y1-ww*, for white pericarp and white glume, and *Y1-cs*, for variegated pericarp and variegated glumes (Fig. 2).

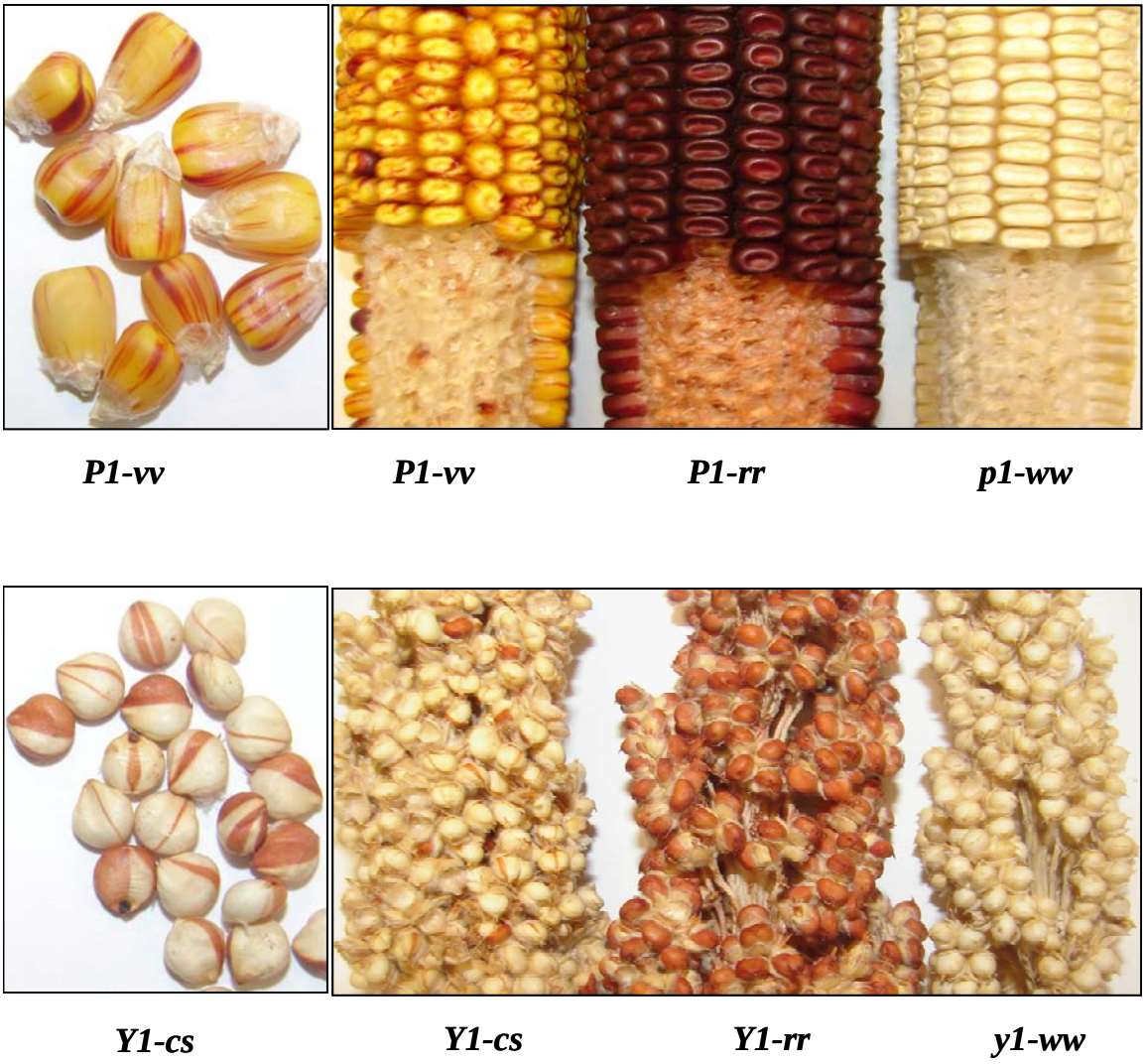

Figure 2 Phlobaphene pigmentation phenotypes of maize ears and sorghum heads carrying different *p1* and *y1* alleles respectively.

1.9 Sorghum phytoalexins

The major phytoalexin compounds in sorghum belong to the rare flavonoid 3-deoxyanthocyanidin class. Sorghum 3-deoxyanthocyanidins have been considered as phytoalexins because of their absence in the uninoculated tissues, their linear and rapid accumulation in response to fungal infection, and their antifungal activity (Nicholson et al., 1987; 1988; Snyder and Nicholson, 1990). These compounds are not commonly present in higher plants and are essentially present as aglycons. They are synthesized as a site specific response to pathogenic and non pathogenic fungi such as *Colletotrichum. sublineolum* and *Cochliobolus heterostrophus* respectively. Sorghum phytoalexins have been characterized and identified as: luteolinidin, apigeninidin, and their derivatives (Nicholson et al., 1987; Hipskind et al., 1990; Snyder and Nicholson, 1990; Lo et al., 1996).

1.9.1 Sorghum 3-deoxyanthocyanidins play an important role in defense against anthracnose disease:

Studies with resistant and susceptible sorghum cultivars demonstrated that resistant cultivars showed faster and rapid accumulation of phytoalexin than the susceptible ones (Lo et al., 1999). Further, the resistant cultivar that accumulated a

significant amount of 3-deoxyanthocyanidins by 36 hour post inoculation (hpi) showed a hypersensitive-response-like phenotype indicated by restricted red pigments in the leaf lamina. In addition, distortion of fungal hyphae was observed by 48 h and fungus progress was completely restricted by 72 hpi. On the other hand, susceptible cultivars showed typical anthracnose disease symptoms which appeared as black spots (acervuli) on the leaf blade indicating the intensive colonization of the fungus (Nicholson et al., 1987; Tenkouano et al., 1998; Lo et al., 1999).

1.9.2 Properties of sorghum 3-deoxyanthocyanidins phytoalexins

Sorghum 3-deoxyanthocyanidins have strong hydrophobic properties and are soluble in less hydrophilic solvents such as methanol (Nielsen et al., 2004). They are more stable in acidic solutions than most known plant anthocyanin and can be extracted in many organic solvents; however the acidified methanol is preferable because it effectively maintains their chemical properties. In addition, these compounds are more efficient in light absorption and more stable at higher pH than normal anthocyanidins (Awika et al., 2004).

1.9.3 Cytological effects of sorghum phytoalexins on fungal and plant tissues.

Sorghum phytoalexins cause many cytological abnormalities in the fungus and the plant cells as well. 3-deoxyanthocyanidins reduced the growth of the germinating germ

tube of both *C. graminicola* and *C. heterostrophus in vitro* (Nicholson et al., 1987). *In vivo*, 3-deoxyanthocyanidins accumulated at the site of fungal spore germination and resulted in disruption, dehydration, and death of the germ tube (Snyder and Nicholson, 1990; Aguero et al., 2002). In addition, the authors observed that phytoalexins accumulated at sites of fungal spore germination and they suggested these sites as the targets of phytoalexins action where they get trapped in the extracellular mucilage released from the fungal spore during germination (Aguero et al., 2002). Here, it is important to point out that the quality and quantity of accumulation of sorghum 3-deoxyanthocyanidins are positively correlated with the time of attempted penetration and interacellular development of the fungal tissues. For example, luteolinidin and luteolinidin 5- methylether, having higher antifungal activity than the rest of the 3-deoxyanthocyanidins, showed a dramatic increase between 24 and 48 hpi (Nicholson et al., 1987; Lo et al., 1996; Wharton and Julian, 1996; Wharton and Nicholson, 2000). This period corresponds to the time of fungal penetration and proliferation of the fungus in the plant tissue (Wharton and Julian, 1996).

On the plant side, the formation of fungal appresorium is usually accompanied by many cytological changes in the underlying epidermal cells. These changes include: increase in the viscosity of cytoplasm, increase in the extensiveness of subcellular organelles such as endoplasmic reticulum and dictyosomes, and appearance of spherical pigmented and colorless inclusions that increase in size with time. These changes end with cell collapse followed by appearance of red pigments on the inter surface of the epidermal cells and later in the fungal tissues. The mesophyll cells underlying the

epidermal cells under attack also show some abnormalities in the chloroplast and vacuole ultrastructure (Snyder and Nicholson, 1990).

1.9.4 Cytoplasmic trafficking, release and possible mode of action of sorghum 3-deoxyanthocyanidins phytoalexins

The synthesis and accumulation of these compounds occurs within colorless inclusions (vesicles, 0.1μm in diameter) in the cytoplasm of epidermal cell under attack. These inclusions then, increase in size, attain orange color, migrate towards the site of fungal penetration, coalesce together forming bigger inclusions (20 μm in diameter). Inclusions become dark red and discharge their content killing both the fungus and the epidermal cell that synthesized them (Snyder and Nicholson, 1990). The mechanisms that control their trafficking in cytoplasm, their color shift and their site-specific discharge at the site of infection are not yet clear. However there are speculations about the presence of a mechanism for self-staking and destruction of these inclusions (Nielsen et al., 2004).

In the inoculated sorghum cells, a number of very interesting phenomena have been reported. These include early migration of the nucleus toward the site of perturbation, polymerization of actin filaments around the nucleus, and movement of 3-deoxyanthocyanidins molecules in rows in the cytoplasmic bridges toward the site of infection. These observations provided unique insights into a possible mechanism for trafficking of sorghum phytoalexins from their site of synthesis to the site of infection. Once sorghum 3-deoxyanthocyanidins molecules are synthesized, they migrate toward

the nucleus via cytoplsmic bridges, most probably in the hydrophilic cytosol because of their strong hydrophobic nature. On their way towards the site of infection, anions released from dissociation of certain acids help to stack these positively charged compounds together forming large size inclusions. As these molecules reach the site of perturbations, they change their color from orange to red, most likely, because of the concomitant decrease in local and subcellular cytoplasmic pH near these sites. This model was supported by *in vitro* experiments showing the same color shift from orange to red after acidification of suspension of 3-deoxyanthocyanidins methanolic extract mixed with water (Nielsen et al., 2004).

At the site of perturbation, the accumulated inclusions physically interact with the inner layer of plasma membrane and disintegrate its lipid bi-layer leading to whole cell collapse. Consequently, the 3-deoxyanthocyanidins inclusions are liberated in the apoplasm and get trapped in the partially hydrolyzed cell wall materials, fungal mucilage, and other fungal structure. In fact, addition of luteolinidins and other 3-deoxyanthocyanidins to artificial liposomes resulted in decomposition of their lipid bi-layer. In addition, supplementing 3-deoxyanthocyanidins to sorghum mesophyl protoplasts maintained in osmotically adjusted solution led to collapse of these protoplasts and accumulation of the 3-deoxyanthocyanidins within protoplast remnants (Nielsen et al., 2004). Within fungal tissues, 3-deoxyanthocyanidins attack the lipid-bi-layer in the fungal membranes at the tip of the hyphae, accumulate within fungal cells and eventually lead to collapse of fungal cells as a mechanism of toxicity.

It is important to point out that the mechanisms of release of sorghum 3-deoxyanthocyanidins at the site of perturbation differ from those leading to the release of other secondary metabolites**.** In general, the transport of secondary metabolites from their site of synthesis to their site of action occurs either via vesicle mediated transport or through a membrane spanning structure. Neither of these two transport mechanisms has self-destruction properties. In addition, the cells that produce these compounds, most probably, remain intact (no cell collapse) and liberate their content to the apoplast (Belanger and Quatrano, 2000). This does not seem to be the case in the release of sorghum 3-deoxyanthocyanidins because collapse of epidermal cells is an unavoidable event and is concomitant with liberation of sorghum 3-deoxyanthocyanidins in the apoplast, and fungal death. Furthermore, sorghum 3-deoxyanthocyanidins inclusions seem to have self destruction mechanism (Nielsen et al., 2004).

1.10 Genetics and Biochemistry of sorghum phytoalexins biosynthesis

Since the discovery of sorghum 3-deoxyanthocyanidins phytoalexins, the mechanism of their biosynthesis has long remained unclear. Because of their flavonoid structure, these compounds are suggested to be products of flavonoid pathway. The synthesis of sorghum phytoalexins most likely occurs via *de novo* synthesis since neither the presumed intermediates nor the phytalexins themselves are constitutively synthesized in sorghum tissues (Hipskind et al., 1996; Weiergang et al., 1996; Boddu et al., 2005) (Fig 1).

The structural similarities between sorghum 3-deoxyanthocyanidins and flavan-4-ols, the precursor of phlobaphene pigments, and the reported concomitant induction of phenylalanine ammonia lyase and chalcone synthase expression during their synthesis (Lo and Nicholson, 1998) led researchers to hypothesize that "the biosynthesis of sorghum phytoalexins may involve activation of part or the entire suite of genes necessary for biosynthesis of phlobaphene". Naringenin and eriodictyol have been suggested as potential intermediates in the putative pathway (Stich and Forkmann, 1988; Hipskind et al., 1996; Halbwirth et al., 2003).

The biosynthesis of 3-deoxyanthocyanidins has been targeted, at the biochemical level, in different systems and sorghum as well. Incubation of C^{14} labeled narigenin and eriodictyol with an enzyme preparation from *Sinningia cardinalis* that contains 3-deoxyanthocyanidins naturally in their petals, led to formation of apiferol and luteoferol respectively (Stich and Forkmann, 1988). The authors reported that the carbonyl groups in the flavanons are reduced in a NADPH-dependant reaction catalyzed by a reductase enzyme. These and other authors suggested that flavan 4-ols are then converted to their corresponding 3-deoxyanthocyanidins, most probably via enzymatic activity of anthocyanidin synthase (Stich and Forkmann, 1988; Winefield et al., 2005).

In sorghum, feeding etiolated seedlings with C^{14} phenylalanin followed by inoculation with the fungus led to the incorporation of radiolabelled phenyalanin into 3-deoxyanthocyanidins, 3-hydroxanthocyanidins and many other unknown phenolic compounds. The sequence of appearance of sorghum 3-deoxyanthocyanidins in response to fungal inoculation has been investigated. Sorghum seedlings respond to fungal infection by production of apigeninidin (10 hpi), the caffeic acid ester of arabinosyl 5-O-

apigeninidin and luteolinidin (14 hpi), and luteolinidin 5-methylether and apigeninidin 7-methylether (18 hpi) (Wharton and Nicholson, 2000). The sequence of synthesis of sorghum phytoalexins allowed speculation about the possible biosynthetic pathway of these compounds. For example, since luteolinidin differs from apigeninidin by *o*-dihydroxylation of the B ring, one speculation is that luteolinidin might be synthesized from apigeninidin by the action of a flavonoid 3' hydroxlase (Boddu et al., 2005). Here, it is worth mentioning that F3'H also has a wide substrate range including narigenin. Another possibility is that F3'H can also drive hydroxylation of naringenin to eryodictol as an initial step toward luteolinidin formation. One more interesting point is that luteolinidin 5-methylester appeared after luteolinindin and accumulated at low level at 24 hpi then showed a dramatic increase between 24 and 48 hpi. These facts might indicate that luteolinidin 5-methylether is synthesized from luteolinidin by an O-methylation step (Wharton and Nicholson, 2000).

Results obtained from sorghum and other systems added a considerable knowledge about the possible mechanisms that could lead to formation of these compounds. However, information about the molecular mechanisms that control flavonoids pathway in cells under fungal attack is still lacking. Interestingly, sorghum lines that have different allelic constitution of *y1* showed different capabilities in synthesizing sorghum 3-deoxyanthocyanidins in response to fungal infection (Chopra et al., 2002). These findings suggest that the biosynthesis of sorghum 3-deoxyanthocyanidins might be under the control of *y1* gene and further confirmation is need.

1.11 Comparative analysis of 3-deoxyanthocyanidin phytoalexins in sorghum and maize

Sorghum and maize mesocotyls respond to light illumination by synthesizing mono and dimalonyl-O-cyanidin glycosides. In addition, the two species activate similar group of structural and regulatory genes during phlobaphenes formation. These results indicate that the flavonoid pathways in both sorghum and maize seem to be under the control of similar suite of structural and regulatory genes. Interestingly, these two species respond differently to fungal challenges. Sorghum responds with synthesis of 3-deoxyanthocyanidins phytoalexins. However, maize lines carrying functional regulatory and structural genes necessary for the production of anthocyanins and phlobaphenes did not synthesize any 3-deoxyanthocyanidins in response to *C. heterostrophus* infection.In addition, the extracts of the inoculated mesocotyls of these lines contained unknown compound of 225 m/z which was also detected in sorghum extracts. With the exclusion of *chs*, neither of the maize flavonoid regulatory nor the structural genes showed induction after fungal infection (Hipskind et al., 1996).

One interesting question to investigate is why does the flavonoids pathway exhibit functional diversity between closely related species like sorghum and maize in response to fungal attack? Modifications of transcription factors have been suggested to be the reason behind many of the reported functional diversity in a number of metabolic pathways (Broun, 2004; Boddu et al., 2006). Thus, one possibility of the differential response of flavonoid pathway in sorghum and maize might be because of the probable evolutionary modifications that have happed in *p1* and *y1*. In fact, molecular analysis of

these two genes indicated that they have a high level of homology (92%) in the coding sequence but very poor homology in the regulatory regions (Boddu et al., 2006). These findings suggest that the biosynthesis of phlobaphene in sorghum leaves and the fungal-induced synthesis of 3-deoxyanthocyanidins in sorghum might be a property of the *y1* promoter. This hypothesis can be tested directly by investigating the heterologus expression of *y1* in maize and investigate the ability to engineer flavan 4-ols, 3-deoxyanthocyanidins, and other flavonoids in maize leaves.

1.12 Co-induction of signaling compounds during sorghum phytoalexins

In general, synthesis of active defense-related compounds involves a number of basic cellular processes including: 1) perception of an extra- or intracellular signal by receptor (s) on the plasmalemma or endomembranes; 2) initiation of a signal transduction cascade that leads to induction of transcription factors; 3) induction of transcription of the structural genes and production of active enzymes; and 4) production of targeted plant defense compounds (Zhao et al., 2005).

The onset of plant defense responses is usually coordinated with activation of a range of defense-related pathways that are mediated by global signaling compounds such as salicylic acid (SA), jasmonic acid (JA), auxin (IAA), abscisic acid (ABA), and reactive oxygen species (ROS). For example, Infection of citrus petals with *Colletotrichum acutatum* induced accumulation of SA, JA, IAA, and ethylene (Lahey et al., 2004). In addition, ABA has been demonstrated to play a role in monitoring levels of ROS probably via mediating levels of antioxidant intermediates and activities of antioxidant

enzymes such as catalase, ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Jiang and Zhang, 2001; 2002).

SA has been considered as a key endogenous signaling molecule in the induction of local and systemic resistance in response to several pathogens (Ryals et al., 1996 ; Durner et al., 1997). The level of SA increased significantly in inoculated cucumber leaves and in tissues located at a distance from infection sites in response to infection with anthracnose fungus, *Colletotrichum lagenarium* (Kubota and Nishi, 2006). In addition, treatment of cucumber leaves with SA induced synthesis of antifungal flavonoid compounds (Wang et al., 2005).

Monocots have relatively high endogenous levels of SA. SA levels in rice are not affected significantly in response to pathogen infection (Silverman et al., 1995). Interestingly, the exogenous application of SA leads to induction and accumulation of H_2O_2 which is also induced in response to fungal infection. This indicates that the induction of H_2O_2 may require a higher level of SA or there is a common link between SA and plant's response to pathogen (Ganesan and Thomas, 2001).

JA has been shown to play an effective role in induction of plant defense mechanisms. In rice, JA induces biosynthesis of the flavonoid phytoalexin sakuranetin (Rakwal et al., 1996). It induces SAR, and stimulates expression of a MYB transcription factor, *JAmyb,* in response to *Pyricularia grisea* (Lee et al., 2001). These authors also reported that SA reduces the expression of *JAmyb*. JA, SA, and H_2O_2 have been shown to induce rice pathogenesis related protein, JIOsPR10, while ABA and ethylene might have a suppression effect (Silverman et al., 1995).

Both synergistic and antagonistic interactions between signaling pathways mediated by these compounds have been demonstrated (Bostock, 2005). These signaling molecules might control expression of plant defense pathways at the transcription level by controlling the transcription factors acting in these pathways. For instance, SA has been shown to induce a plant transcription factor WRKY70, while JA suppresses its expression. Interestingly, over expression of WRKY70 caused constitutive expression of SA dependent genes (*PR1, PR2,* and *PR5*), while its suppression activates JA dependent gene expression (Li et al., 2004).

In sorghum, the exogenous application of SA and JA induces expression of phenylpropanoids and flavonoid genes encoding phenylalanine ammonia lyase (*pal*), cinnamate-4-hydroxylase (*c4h*), chalcone synthase (*chs*), chalcone isomerase (*chi*), and dihydroflavanol reductase (*dfr*) (Salzman et al., 2005). In addition, these signaling compounds induce expression of many PR proteins including PR1, PR2, PR3, PR5, and PR10. Further, cellular oxidative state related genes have also been shown to be induced by SA and JA. These include genes encoding putative peroxidases, oxalate oxidase, and glutathione S-transferase. Interestingly, NADPH oxidase which is considered as a major producer of H_2O_2, was unaffected by SA, while its expression was induced by JA (Salzman et al., 2005).

Despite the fact that sorghum 3-deoxyanthocyanidin phytoalexins were characterized more than one decade ago, very little effort has been focused on dissection of the signal transduction cascade that leads to their induction. To the best of our knowledge, biochemical analysis of the concomitant changes in the levels of global

signaling compounds that lead to or are co-induced with synthesis of sorghum phytoalexins has not been previously reported**.**

Research objectives and possible outcome

Although sorghum 3-deoxyanthocuanidin phytoalexins are frequently induced in response to fungal infection, the transcriptional regulation of their biosynthesis is poorly understood. In addition, very scarce studies regarding the coordinated physiological and biochemical processes during their biosynthesis are available. It is quite clear that sorghum near-isogenic lines with differential capabilities of phytoalexins biosynthesis are needed for genetic studies. Comparing phytoalexins biosynthesis and anthracnose reaction phenotypes in these isogenic lines will be helpful not only to narrow down genes that are most likely involved in 3-deoxyanthocyanidin biosynthesis but also to better understand their role in defense against anthracnose fungus.

A study on the transcriptional regulation of sorghum phytoalexins would include identifying the transcription factor that regulates the flavonoid genes to produce these defense compounds under fungal attack. An ideal candidate for this function would be the sorghum *yellow seed1* (*y1*). Thus, one of my research goals is to address the question: Is *y1* necessary for biosynthesis of sorghum phytoalexins? To answer this question, we developed and utilized two near isogenic sorghum lines that share the genetic background but differ in the allelic constitution of *y1* locus. We employed a number of molecular, biochemical and pathological techniques to evaluate the biosynthesis of sorghum

phytoalexins in these two lines. The results of this work (see Chapter 2) indicated that *y1*, in deed, is required for synthesis of sorghum phytoalexins.

The close genetic relationship between sorghum and maize, the high level of synteny between their genomes, and their different capabilities in synthesizing 3-deoxyanthocyanidins in response to fungal challenges, made maize a perfect system to study the heterologus expression of sorghum *y1*. To achieve this goal, we have developed transgenic maize lines that express different *y1* constructs. The generation, molecular, biochemical, phytopathological evaluation of these maize lines will be described in details in Chapter three.

Induction of defense secondary metabolites is controlled by an intricate signal transduction network. A large body of research established that pathogen attack induces accumulation of global signaling compounds such as SA, JA, IAA, ABA, and reactive oxygen species. These compounds regulate the cross-talk among the plant metabolic pathway. The phenypropanoid and flavonoid genes are reported to be responsive to SA and JA; however, the information about the level of these compounds during sorghum-fungus interaction is lacking. Therefore, chapter 4 of my thesis is dedicated to the study of the possible induction of a number of global signaling compounds at different time intervals after *C. heterostrophus* inoculation.

The results of my research will hopefully aid in a better understanding of the physiological, molecular and biochemical aspects of phytoalexins synthesis. It will also aid in understanding the functional significance of flavonoids in adaptation of plants to biotic stress. Further, it might help in generating crops with better ability to withstand a

wide array of pathogen challenges. This will reduce the need for excessive chemicals and pesticides that have been the cause of soil, water, and air pollution.

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Chapter 2

A null mutant of a MYB gene *yellow seed1* shows loss of 3-deoxyanthocyanidin phytoalexins and susceptibility to anthracnose fungus in *Sorghum bicolor*

Submitted to Molecular Plant-Microbe Interactions

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Abstract

Phytoalexins are low molecular weight compounds synthesized by plants during biotic and abiotic stress. In sorghum (*Sorghum bicolor*), a set of compounds belonging to the 3-deoxyanthocyanidin class that include luteolinidin, apigeninidin and their derivatives are produced at the site of infection in response to pathogenic and non-pathogenic fungi. These compounds have structural similarities to flavan-4-ols which are precursors to brick red phlobaphene pigments that accumulate during plant development in sorghum seed pericarp and other tissues. We have previously shown that the sorghum *yellow seed1* (*y1*) encodes a MYB type of transcription factor that regulates phlobaphenes' biosynthesis in sorghum. We have now isolated sorghum mutants carrying *y1* loss of function alleles that do not accumulate flavan-4-ols. These mutants were isolated from a mutable *y1* allele that carries the *candystripe1* transposon within the second intron of the *y1*. These *y1* null alleles have a partial deletion within the *y1* sequence. In addition to the absence of flavan-4-ols and phlobaphenes, the null allele, designated as *y1-ww-1* (white pericarp, white glumes), do not accumulates 3-deoxyanthocyanidin phytoalexins when challenged with *Cochliobolus heterostrophus.* Absence of 3-deoxyanthocyanidin phytoalexin synthesis in the *y1-ww-1* background is further correlated with its enhanced susceptibility to *Colletotrichum sublineolum*, a fungus that causes anthracnose disease in sorghum. In fungal inoculated *y1-ww-1* seedlings, we did not detect steady state transcripts of *y1* and flavonoid structural genes shown to be regulated by *y1*. Our results indicate that the sorghum 3-deoxyanthocyanidin phytoalexins and phlobaphenes are synthesized through a common pathway under the regulatory control of the *y1* gene.

Introduction

During attempted invasion by pathogens, plants defend themselves via induced and systemic acquired resistance mechanisms. These mechanisms include biosynthesis of cell wall strengthening compounds like lignins (Bruce and West, 1989), flavonoids and isoflavonoids (Dixon and Steele, 1999), induction of PR proteins and hydrolytic enzymes such as chitinases and glucanases (Kombrink et al., 1988; Mehdy, 1994), and biosynthesis of phytoalexins (Dixon and Lamb, 1990; Hammerschmidt, 1999). Phytoalexins, low-molecular-weight anti-microbial compounds, are structurally diverse plant secondary metabolites. Some examples of phytoalexins include isoflavonoids and pterocarpans in legumes (Paxton, 1995), sulphur containing indole phytoalexins found in cruciferous plants (Tsuji et al., 1992), sesquiterpenoid phytoalexins produced in solanaceous plants (Brooks and Watson, 1991), and coumarin phytoalexins in umbelliferous plants (Knogge et al., 1987). In rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*), flavonoid compounds have been shown to act as phytoalexins against *Magnaporthe grisea* and *Colletotrichum spp.,* respectively (Snyder and Nicholson, 1990; Kodama et al., 1992).

In sorghum leaves, phytoalexin induction can be stimulated by inoculation with either a pathogenic (*Colletotrichum sublineolum*) or a non-pathogenic (*Cochliobolus heterostrophus*) fungus (Nicholson et al., 1987). In the laboratory, etiolated seedlings rapidly respond to the challenge of *Cochliobolus heterostrophus* by producing reddish-brown pigments. These pigments are induced in the epidermal cells at the site of attempted fungal infection (Snyder and Nicholson, 1990). Biochemical characterization of pigments allowed the identification of 3-deoxyanthocyanidins which include

luteolinidin, 5-methoxy-luteolinidin, apigeninidin, caffeic acid ester of arabinosyl 5-O-apigeninidin and 7-methoxyapigeninidin (Snyder and Nicholson, 1990; Lo et al., 1996; Wharton and Nicholson, 2000). The 3-deoxyanthocyanidins are members of 3-deoxyflavonoids which are also precursors of phlobaphene pigments. Phlobaphenes accumulate in the kernel pericarp and other maize (*Zea mays*) and sorghum tissues predominantly at the time of physiological maturity (Kambal and Bate-Smith, 1976; Styles and Ceska, 1977). Phlobaphene pigments have been proposed to arise from polymerization of flavan-4-ols (Styles and Ceska, 1989) and a parallel pathway has been suggested for the biosynthesis of 3-deoxyanthocyanidin phytoalexins (Fig. 1) (Lo and Nicholson, 1998; Boddu et al., 2004; 2005).

In many plant-pathogen interactions, the role of phytoalexins in inhibiting pathogen growth and development in host tissues is poorly understood. One of the approaches has been the use of phytoalexin deficient mutants (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997; Hammerschmidt, 1999; 2003). In *Arabidopsis thaliana*, many phytoalexin-deficient (*pad*) mutants have been studied. Genetic characterization of the *pad3* mutant indicated that the phytoalexin camalexin plays an important role in defense against *Alternaria braccicicola* but not against *Botrytis cinerea* (Thomma et al., 1999).

The contribution of 3-deoxyanthocyanidins to sorghum resistance against a pathogenic fungus has been investigated by comparing the ability of different cultivars to produce phytoalexins and then relating any observed variation to disease response (Wharton and Julian, 1996; Tenkouano et al., 1998; Lo et al., 1999). These studies

indicated that phytoalexin production in the resistant cultivars was not only more rapid but also more intense than in the susceptible cultivars. In addition, phytoalexins accumulation was associated with the distortion of fungal hyphae and restriction of further fungal proliferation in the resistant cultivars. On the other hand, intensive colonization of the tissue by the fungus was reported on the leaf blade of susceptible sorghum plants (Nicholson et al., 1987; Tenkouano et al., 1998; Lo et al., 1999). Although the potential contribution of sorghum 3-deoxyanthocyanidins to sorghum disease resistance is well established, the genetic and molecular mechanisms controlling their biosynthesis is far from clear.

In general, phytoalexins are produced via complex biosynthetic pathways that include the stepwise activation of many structural genes which are often regulated by transcription factors. These transcription factors are responsive to signal transduction cascades which are usually activated upon recognition of environmental cues (Nessler, 1994; Chen and Zhu, 2004). In the current work, isolation and characterization of phytoalexin deficient mutants of sorghum has been performed to dissect the phytoalexin biosynthetic pathway. The set of mutants analyzed here are in the *yellow seed1* (*y1*) gene which encodes a MYB transcription factor. We tested the hypothesis that the *y1* gene is required for the biosynthesis of both phlobaphenes and 3-deoxyanthocyanidin phytoalexins in sorghum. The potential contribution of 3-deoxyanthocyanidins in protecting sorghum plants against anthracnose disease was also confirmed.

Materials and Methods

Sorghum and fungal stocks

We have used the maize genetics nomenclature to name and describe sorghum **[***Sorghum bicolor* (L.) Moench**]** *y1* alleles: Locus or gene is designated as *y1,* while alleles are shown as dominant (functional) or recessive (non-functional) followed by a two letter suffix representing pericarp and glume pigmentation. For example, *Y1-rr* specifies red pericarp and red glumes while *y1-ww* conditions white pericarp and white glumes. The *Y1-rr-3* and *y1-ww-1* alleles used in this study originated from a common progenitor line [CS8110419] that carries a mutable *Y1-cs-30* (candystripe) allele (Chopra et al., 1999). Both *Y1-rr-3* and *y1-ww-1* alleles resulted from spontaneous excision of the *candystripe1* (*cs1*) transposon. The functionally revertant alleles or the *Y1-rr*'s arise with a frequency of approximately 10% and the structure of one such alleles has been described previously (Chopra et al., 1999; Boddu et al., 2004). The non-functional *y1-ww* alleles were obtained with a frequency of 0.1%. Three of the *y1-ww* alleles characterized have similar *cs1* insertion and deletion events, and the molecular structure of one of the *y1-ww* alleles is described here.

Fungal cultures of *Cochliobolus heterostrophus* **(**anamorph *Bipolaris maydis* [Nisikado and Miyake] Shoemaker**)** and *Colletotrichum sublineolum* [P. Henn, Kabat and Bubak] were maintained on potato dextrose agar under constant illumination at 26°C for 7 -10 days. The conidial suspensions were prepared in a tween/water mixture as described previously (Lo and Nicholson, 1998; Lo et al., 1999). Conidial suspensions were filtrated through cheese cloth and diluted to get a concentration of 10^6 spores ml^{-1}.

Plant growth & fungal inoculation conditions

Seeds were surface sterilized for 1 h with 10% commercial bleach, washed in running tap water and then imbibed in sterilized water for 12 h. To get seedlings with uniformly etiolated mesocotyls, seeds were planted in rolls of germination paper and incubated in the dark for 5-7 days at 26°C in a growth chamber. Phytoalexins biosynthesis was induced by inoculation of seedlings with *C. heterostrophus* which is non-pathogenic to sorghum. This fungus was used because of the extremely fast response of induction of phytoalexins during its attempted penetration of sorghum tissues (Lo and Nicholson, 1998; Aguero et al., 2002). For inoculation, the conidial suspension was sprayed onto etiolated seedlings followed by incubation in the dark at 26°C in a growth chamber with 100% relative humidity. For controls, etiolated seedlings were sprayed with mock inoculation medium containing tween/water mixture only. Triplicate samples of mesocotyl tissue were collected at 0, 24, and 36 hours post-inoculation (hpi) from both genotypes as well as at 0, 3, 6, 9, 12, and 24 h from inoculated and control *Y1-rr-3* seedlings. At the time of tissue collection, seedlings were first photographed using a dissection microscope (Nikon SMZ1000) connected with a Nikon digital camera (DXM1200F), excised 5 mm above the point of attachment to the seed and the upper part of seedling was collected. The collected tissues were snap frozen and stored at -80°C for biochemical and gene expression analyses.

A correlation between the biosynthesis of 3-deoxyanthocyanidins and anthracnose disease resistance in the two sorghum stocks was investigated. Seeds were surface sterilized and soaked in water for 12 h and then planted in universal soil mixture in pots

of 30 cm diameter and placed in a growth chamber at 26°C with 16 h of day light and 8 h dark cycle. When plants had reached the four leaf stage the conidial suspension of *Colletotrichum sublineolum*, the causal agent of sorghum anthracnose disease, was applied to the third leaf. After inoculations, relative humidity was maintained at 100% for 12 h to facilitate spore germination and infection. The development of disease symptoms was recorded after 2 and 7 days and induced pigments that accumulated around the sites of infection were collected at 72 hpi for biochemical analysis.

Spectrophotometery & Thin Layer Chromatography

To detect the presence of sorghum 3-deoxyanthocyanidins in the *Y1-rr-3* and *y1-ww-1* genotypes after fungal inoculation, seedling tissues were harvested and ground into fine powder using a mortar and pestle in liquid nitrogen. One hundred mg of powdered tissues collected at 0, 24, and 36 hpi were incubated in 0.083 % HCl in HPLC grade methanol at 4°C for 24 h. The clear extracts were collected after centrifugation at 10,000 rpm for 10 minutes. Supernatants were screened for the presence of 3-deoxyanthocyanidins by profiling the spectra over a range of 250 to 550 nm using an UV mini-1240 spectrophotometer (Shimadzu Scientific Instruments, Inc. Columbia, MD). Total 3-deoxyanthocyanidin phytoalexin content in the extracts was quantified spectrophotometrically as described previously (Nicholson et al., 1987; Yamaoka et al., 1990). Briefly, absorbance of the methanolic extracts was measured at 480 nm using 1 cm wide quartz curvet in a UV mini-1240 spectrophotometer. The concentration of

phytoalexins in the methanolic extract was expressed as μM of phytoalexins based on the Lambert-Beer Law using the molar extinction coefficient of luteolinidin (13800 $M^{-1}cm^{-1}$) (Aguero et al., 2002).

Equivalent amounts of the ground tissues were extracted in HPLC grade methanol and the extracts were clarified by centrifugation at 14,000 rpm for 10 min. Equal aliquots of the clear extracts were loaded on to a 20 x 20 cellulose plate (Analtech, Inc. Newark, DE). TLC (thin layer chromatography) plates were developed using a mixture of CH_3COOH:HCl:H_2O (30:3:10, v/v) as a mobile solvent. For controls, commercially available 10 ng/μl apigeninidin and luteolinidin (Extrasynthese, Genay Cedex, France) were loaded in adjacent lanes for comparison and identification of compounds.

HPLC and LC/MS analysis

For HPLC analysis, 20 mg of tissue was placed in 1 ml of HPLC grade methanol and compounds were allowed to leach at 4°C for 24 h in the dark. Extracts were collected by centrifugation at 1,000 rpm. Additional contaminating large particles were removed by filtration through 0.45 um Acrodisc LC 13 mm syringe filters (Gelman Laboratory, Ann Arbor, MI). These extracts were then transferred to fresh tubes, and evaporated down to 100 μl. To separate compounds using HPLC, two reversed phase C18 columns connected in tandem (Supelco, Bellefonte, PA) were used and a 50 μl sample was applied to the column fitted on a Waters 600 HPLC separator with a Waters 900 detector system. Spectral measurements were taken over a wavelength range of 230-

550 nm, which is known to detect all flavonoid compounds (Grotewold et al., 1998). Pure luteolinidin and apigeninidin (Extrasynthese, Genay Cedex, France) were dissolved in HPLC grade methanol and used as standards. Detection of 3-deoxyanthocyanidins was carried out at 480 nm as described previously (Snyder and Nicholson, 1990; Lo and Nicholson, 1998).

LC-MS analyses were carried out using a Quattro II mass spectrometer (Micromass, Beverly, MA) interfaced with a Shimadzu LC10ADvp pump. Compounds containing 3-deoxyanthocyanidins were separated on a 1X 150 mm BetaBasic C18 column with a solvent gradient of water + 0.15 % formic acid (solvent A) and methanol (solvent B) and a flow rate of 0.05 ml min^{-1} delivered using a pre-injection split. The program of solvent composition was established as: 0-5 min (99% A; 1% B), followed by a linear gradient to 50% A; 50% B at 12 min, another linear gradient to 100% of solvent B at 20 min, with a hold at 100% B until 25 min. Electro-spray ionization was carried out in the positive ions mode for detection of 3-deoxyanthocyanidin compounds.

DNA gel blot analysis

Plant genomic DNA was extracted from sorghum seedlings following the standard method (Saghai-Maroof et al., 1984). DNA was digested using restriction enzymes, reagents and reaction conditions from Promega (Madison, WI). DNA digests were fractionated on 0.8% agarose gels and gels were blotted onto a nylon membrane by using a standard protocol of capillary transfer (Sambrook and Russell, 2001). DNA was cross-linked to the membrane through a UV cross linker (TL-2000 Ultraviolet trans-

linker). DNA gel blots were pre-hybridized for 6 h in a hybridization mixture containing 10% dextran sulfate, 1M NaCl, 1% SDS, 10mM Tris-HCl, and 0.25 mg ml^{-1} sheared salmon sperm DNA. Blots were hybridized at 65^{o}C for 24 h in fresh pre-hybridization buffer along with ^{32}P labeled and denatured DNA probes. Blots were washed twice at 65^{o}C in a solution of 0.5% SSC and 0.1% SDS. Blots were wiped semi-dry and then exposed to X-OMAT films (KODAK, Rochester, NY) for 2-3 days prior to developing. DNA gel blots were washed in a boiling solution of 0.1% SDS before re-hybridization.

RNA gel blot analysis

Total RNA was isolated from inoculated sorghum seedlings collected at various time points using TriReagent according to the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH). Five micrograms of total RNA per sample was fractionated on 1.2 % denaturing gels containing 5 % (v/v) formaldehyde. After electrophoresis, gels were rinsed in DEPC treated water and blotted onto a nylon membrane (Osmonics, Inc., Minnetonka, MN) by standard capillary transfer. RNA was cross-linked to the membrane using a UV cross-linker (TL-2000 Ultraviolet trans-linker). RNA blots were hybridized with ^{32}P-labeled individual DNA probes for *y1, chs1, chi1, f3'h3* and *dfr1* genes. Slot blots were prepared using denatured DNA fragments of different genes under study. For slot blot hybridizations, RNA samples (24 hpi) were reverse transcribed and the first cDNA strand was used as a probe. During the reverse transcription reaction, ^{32}P-dCTP was used as the radioactive label. All blots were exposed to X-OMAT x-ray films (KODAK, Rochester, NY) for 3 days prior to developing. A

boiling solution of 0.1% SDS was used for stripping the blot before the next hybridization.

Plasmids and probes used for DNA and RNA hybridizations.

All plasmid DNA was isolated using the Maxi-prep DNA isolation kit (Promega, Madison, WI, USA). Positions of *y1* gene probes used for gel blot hybridizations are shown in Figure 3A. The y1-F-3 probe is a DNA fragment of 2.2 kb size corresponding to the intron 2 region and was prepared by digesting plasmid DNA pY1-22 with *Xho*I-*Hin*dIII. The y1-cDNA probe was prepared by PCR amplification of a part of y1-cDNA including exon 1, exon 2 and a 70 bp region of exon 3 using Y1-F1, 5'-ACACACTGCGAGCTGAGAG-3' and Y1-R3, 5'-CGAGTTCCAGTA GTTCTTGATC-3' as forward and reverse primers, respectively; see Figure 3A for the position of primers. Flavonoid structural gene probes consisted of DNA fragments obtained from plasmids pZMC2 containing a maize *c2* cDNA (Wienand et al., 1986), plasmid pZMA1 with a maize *dfr1* cDNA (Schwarz-Sommer et al., 1987), p*Chi1* containing maize *chi1* cDNA obtained from Dr. Erich Grotewold, Ohio State University and pZMF3'H3 containing a sorghum *f3'h3* gene (Boddu et al., 2004). DNA fragments of interest were purified from gels using UltraClean GelSpin kit (Mo Bio Laboratories, Inc, CA). For all probe preparations, the [α-^{32}P] dCTP was used for random prime labeling of DNA fragments using prime-a-gene kit (Promega, Madison, WI, USA).

PCR analysis

PCR analysis was performed on genomic DNA extracted from seedlings of *Y1-rr-3*, *Y1-cs-30,* and *y1-ww-1.* Additional partially characterized *white pericarp, white glume* alleles included *y1-ww-4*, *y1-ww-264* and *y1-ww-971* (F. Ibraheem and S. Chopra, unpublished). Forward primer Y1-F1, 5'-ACACACTGCGAGCTGAGAG-3' and reverse primer Y1-R1, 5'-GGATCAACTACCTTCGGGCCGACGT-3' were used for amplification of a *y1*-DNA fragment corresponding to regions of exon 1, intron 1, and a part of exon 2 (primer positions shown in Fig. 3A). In addition, Y1-F2 5'-CAAGAACTACTGGAATTCGCACCT-3' and Y1-R2, 5'-CTTCTTCACATGTAC TGTACT-3' primers were used for amplification within exon 3. All genomic PCR reactions were performed using standard conditions with annealing temperature of 58^{o}C and 30 cycles of amplification.

Results

Null *y1* alleles have internal deletions

Genetic tests showed that sorghum mutant stocks with white pericarp and white glumes phenotype (Fig. 2A) segregated as recessive alleles of *y1* and thus were designated as *y1-ww* alleles. Several generations of selfing established that *y1-ww-1* is a stable mutant line. To characterize the cause of non-functionality of the *y1-ww-1* allele, DNA gel blot analysis was carried out using genomic DNA extracted from *Y1-rr-3*, *Y1-cs-30*, and *y1-ww-1* seedlings. Genomic DNA was digested with several restriction enzymes and gel blots were hybridized with DNA fragment, F-3 of *y1* as a probe. This fragment corresponds to the intron 2 region of *y1* and it is highly specific for the *y1* gene

(Boddu et al., 2006). Results of DNA gel blot hybridizations are shown in Figure 2B. In *Bam*HI digest of *Y1-cs-30*, the F-3 probe hybridized to two bands of approximately 7.5 kb and 4.9 kb sizes. The 7.5 kb band originates from digestion of *Bam*HI sites at positions 2633 and 3911 within *y1* and the *cs1* transposon, respectively. The 4.9 kb band is generated due to *Bam*HI sites at positions 2633 and 7542 in *y1* sequence. In *Bam*HI digest of *Y1-rr-3* DNA, the 7.5 kb band is not present because of the absence of *cs1* element. Interestingly, the F-3 probe did not hybridize to any genomic DNA fragments in *y1-ww-1 Bam*HI digest. Similarly, bands of expected size were observed in *Hin*dIII, *Sca*I, and *Sal*I digests of *Y1-cs-30* and *Y1-rr-3* DNA with fragment F-3. This probe did not detect any homologous sequences in *y1-ww-1* DNA digests indicating that fragments F-3 may either be deleted or altered. To test whether there is any deletion in the *y1* coding region of the *y1-ww-1* line, the DNA gel blot was hybridized with the 5' region of the *y1* cDNA (Fig. 2B). In *Y1-cs-30* and *Y1-rr-3 Bam*HI digests, the probe hybridized to bands of approximate sizes of 5.0 kb and 4.0 kb representing *y1* and *y2* genes, respectively; *y2* is a pseudogene that is 8.5 kbp 3' to the *y1* gene sequence (Boddu et al., 2006). Interestingly, the cDNA probe hybridized only to the 4 kb *Bam*HI band (*y2* specific) in *y1-ww-1*. In the *Hin*dIII digest, the 6.0 kb *y1*-specific band is absent in *y1-ww-1*. Similarly, in *Sca*I digest, the 1.9 kb band belonging to *y1* is also absent. This is indicated by low intensity of this band in *y1-ww-1* lane as compared to *y1-rr-3* and *y1-cs-30,* both of which have *y1* and *y2* specific bands of 1.9 kb. *Sal*I digest hybridization results could not be resolved very well because of large fragment sizes. These results established that the *y1-ww-1* allele has an internal deletion.

To further characterize the extent of the deletion within the *y1* sequence of *y1-ww-1*, PCR analysis was performed using two sets of primers (Fig. 3A). Primers located in exon 1 and 2 gave rise to expected PCR products of 846 bp in both *Y1-cs-30* (Fig. 3B, lane 1) and *Y1-rr-3* (lane 2). *y1-ww-1* and other deletion alleles of *y1* did not show the presence of this expected PCR product. However, primers corresponding to exon 3 detected *y1* (1016 bp) and *y2* (821 bp) specific PCR products. These results together with DNA gel blot hybridization indicated that *y1* is either deleted or significantly modified in *y1-ww* alleles. The extent of sequence alterations corresponds to exon1, intron1, exon2 and intron 2, while exon3 remained intact. The molecular characterizations indicated that the *y1* nulls result from the improper excision of the *cs1* transposon from *y1* (data not shown). The mechanism leading to an interstitial deletion while leaving a fractured transposon within the *y1* sequence is currently under investigation. The null *y1-ww-1* allele is stably inherited because of the inability of the fractured *cs1* transposon to excise out of the *y1* (data not shown).

Fungal induced 3-deoxyanthocyanidins are not synthesized in *y1-ww-1* mesocotyls

Based on the proposed role of *y1* in regulation of flavonoid biosynthesis, we tested the hypothesis that a functional *y1* gene is required for the synthesis of 3-deoxyanthocyanidin phytoalexins. Dark grown etiolated seedlings of null *y1-ww-1* and functional *Y1-rr-3* lines were inoculated with *C. heterostrophus.* Samples collected at 24 and 36 hpi showed accumulation of red-brown pigments in *Y1-rr-3* mesocotyls (Fig. 4A). Thin layer chromatographic analysis of *Y1-rr-3* extract showed the presence of two bands

that co-migrated with the standards luteolinidin and apigeninidin (Fig. 4B). These bands were not detected in the *y1-ww-1* extract.

The acidified methanol extracts of the inoculated tissues showed absorption spectra characteristic of 3-deoxyanthocyanidins (λ_{max} at 480 nm) (Fig. 5A). Total phytoalexins concentration was measured spectrophotometrically in the acidified methanolic extract at 0, 24, and 36 hpi. Results presented in Figure 5B indicate that in response to fungal infection, sorghum plants carrying a functional *y1* gene accumulated much higher levels of 3-deoxyanthocyanidin compounds than the one with a non-functional *y1-ww-1* allele.

The identity of the compounds was confirmed by HPLC (Fig. 6A). Pure luteolinidin and apigeninidin were used as standards to confirm the retention times of the newly identified compounds. Our results showed that sorghum plants with a functional *y1*gene (*Y1-rr-3*) produced luteolinidin (retention time, ~10 min), apigeninidin (retention time, ~11 min), and 5-methoxyluteolinidin (retention time, ~12.8 min). The null *y1-ww-1* plants produced negligible amounts of unknown compounds. *Y1-rr-3* and *y1-ww-1* extracts were further subjected to LC-MS analysis and the results are shown in Figure 6B. The *Y1-rr-3* extract showed three major and several minor peaks. Major peaks corresponded to the reported 3-deoxyanthocyndins including luteolinidin (271 m/z), methoxy-apigeninidin (269 m/z), and methoxy-luteolinidin (285 m/z). One of the observed minor peaks was for a compound with a mass consistent with that of apigeninidin (255 m/z) while the other minor peaks could not be confirmed. Interestingly, in the *y1-ww-1* extract minor chromatogrms that may correspond to trace amounts of

certain 3-deoxyanthocyanidins or related compounds were observed. In all of the above experiments, extracts of un-inoculated mesocotyls did not show induction of any pigments or 3-deoxyflavonoid compounds (data not shown).

y1 and flavonoid structural genes are coordinately induced after fungal ingress

To test whether the expression of the flavonoid structural genes was dependant on *y1* functionality, total RNA was extracted from inoculated mesocotyls of *Y1-rr-3* and *y1-ww-1* at 24 hpi and slot blotted. The slot blots were hybridized with radioactively labeled first strand cDNA obtained from reverse transcription of this RNA (Fig. 7A). The transcript of *y1* showed a significant level in *Y1-rr-3* while no transcript was observed in *y1-ww-1*. Interestingly, *chs* and *f3'h* expression were also induced after fungal challenges in *Y1-rr-3* but not in *y1-ww-1*. These preliminary results are evidence that *y1* and its candidate targets in the flavonoid pathway are induced after inoculation with *C. heterostrophus* and the differential induction of flavonoid genes correlates with the allelic constitution of the *y1* locus. To confirm these results, *Y1-rr-3* mesocotyls were used to monitor the temporal expression of *y1* transcripts and relate it to that of the expression of structural genes of the flavonoid pathway during the early events of the sorghum-*C. heterostrophus* interaction. Total RNA was isolated at different time points as indicated (see Materials and Methods) and RNA gel blots were hybridized with *y1* and flavonoid structural gene probes (Fig. 7B). *y1* gene transcripts were observed as early as 3 hpi and showed progressive accumulation up to the end of our experimental period. Neither *chs* nor *f3'h* showed any detectable level of expression up to 6 hpi. Their transcripts started to

accumulate by 9 hpi and reached a plateau at 12 hpi with a considerably higher level of *chs* induction than that of *f3'h*. Interestingly, the *dfr* gene exhibited much lower levels of induction at 6 hpi, 12 hpi and then showed a sharp increase at 24 hpi. On the other hand, *chi* showed expression through all time points with higher induction at the latter ones (9-24 hpi). These results indicated that the transcriptional machinery of the flavonoid pathway is active in response to *C. heterostrophus* infection. In addition, our data established that the induction of *y1* expression preceded that of its candidate targets and the expression of this transcription factor and that of the flavonoid structural genes are coordinately regulated during sorghum x fungus interaction.

y1-ww-1 line shows symptoms of anthracnose susceptibility

We investigated the consequences of deficiency in 3-deoxyanthocyanin accumulation on the ability of sorghum plants to restrict pathogen development within sorghum tissues. We tested if the revertant *Y1-rr-3* and mutant derivative *y1-ww-1* would have differential sensitivity to *C. sublineolum,* the causal pathogen of sorghum anthracnose disease. Two weeks old seedlings were inoculated with a suspension of fungal conidia and inoculated seedlings were placed under white light. Disease symptoms were recorded at two and seven days post-inoculation. Forty-eight hours after inoculation, both lines produced reddish-brown spots typical of the hypersensitive-like response at the site of the primary infection (Fig. 8A, panel a; Snyder and Nicholson, 1990). Further, seven days after infection, the *y1-ww-1* line produced leaf lesions, while

the leaves of *Y1-rr-3* line only produced localized red spots (Fig. 8A, panel b). Dark spots representing acervuli of the fungus were observed on the leaves of *y1-ww-1* line and this symptom is typical of a susceptible phenotype (Lo et al., 1999). This result shows that the presence of functional *y1* gene correlates with the resistance to the fungus *C. sublineolum* in sorghum.

Pigments induced at 72 hpi were extracted and subjected to HPLC analysis. HPLC chromatograms from both sorghum lines showed significant difference in their flavonoid profile. Five major peaks with the retention times of 13.63, 14.20, 16.24, 18.40 and 18.75 min appeared in *Y1-rr*-3 extract. Three of these peaks were identified, based on their elution times, as luteolinidin (retention time, 13.63 min), Apigeninidin (retention time, 14.2 min) and 7-methoxy-apigeninidin (retention time, 16.2 min) while the other two peaks could not be identified. In addition, some minor peaks of unknown compounds were also observed in the *Y1-rr-3* extract. Interestingly, all these peaks were absent in the *y1-ww-1* extract and instead two peaks corresponding to unknown compounds were observed. Our results thus provide genetic evidence that a sorghum line that produces 3-deoxyanthocyanidin phytoalexins under the control of functional *y1* gene is resistant while a sibling line that carries a transposon induced deletion of the *y1* gene is susceptible to anthracnose fungus.

Discussion

Phytoalexins are an important component of inducible plant defense mechanisms and their accumulation is tightly controlled both temporally and spatially (Dixon et al.,

1983). Their biosynthesis is induced by different external stimuli such as pathogen attack (Snyder and Nicholson, 1990; Glazebrook and Ausubel, 1994; Dillon et al., 1997; Jung et al., 2005), and UV irradiation (Kodama et al., 1992) as well as exogenous application of elicitors or signaling compounds (Rakwal et al., 1996). Sorghum plants respond to challenges of pathogenic and non-pathogenic fungi by the production of flavonoid phytoalexins belonging to the 3-deoxyanthocyanidin class (Nicholson et al., 1987; Lo et al., 1999) fungal penetration and their chemical structure and antifungal activity have been well established (Nicholson et al., 1987; Snyder and Nicholson, 1990; Weiergang et al., 1996; Nielsen et al., 2004). However, their biosynthetic pathway is still unclear and the reason for that being the absence of well defined sorghum phytoalexin mutants. In Arabidopsis, the use of phytoalexin deficient mutants has been helpful in dissecting the camalexin biosynthetic pathway and testing the contribution of this defense compound to a plant's resistance (Glazebrook and Ausubel, 1994; Hammerschmidt, 2003).

The structural similarities between 3-deoxyanthocyanidin phytoalexins and flavan-4-ols, the precursors of the red phlobaphene pigments found in the pericarps of sorghum and maize, suggest that these compounds might be synthesized via a common or overlapping pathway (Kambal and Bate-Smith, 1976; Stich and Forkmann, 1988; Styles and Ceska, 1989; Schutt and Netzly, 1991; Grotewold et al., 1998; Wharton and Nicholson, 2000; Chopra et al., 2002; Winefield et al., 2005). We have previously followed a genetic approach showing that the biosynthesis of sorghum flavan-4-ols requires an R2R3 MYB transcription factor encoded by *y1* (Boddu et al., 2005; 2006). In the current study, we have used a null allele of the *y1* and investigated its ability to

synthesize 3-deoxyanthocyanidin phytoalexins in response to pathogenic and non-pathogenic fungi. The molecular analysis of the *y1-ww-1* allele indicated that it is non-functional due to an internal deletion. Biochemical analysis indicated that *y1-ww-1* is deficient in 3-deoxyanthocyanidin biosynthesis. Conversely, the functional *Y1-rr-3* allele accumulates all known 3-deoxyanthocyanidins.

Phytoalexin biosynthesis has been shown to be associated with *de novo* protein synthesis of enzymes required for the biosynthesis of these induced compounds (Dixon et al., 1983; Cramer et al., 1985; Berenbaum and Zangerl, 1992; Dixon and Paiva, 1995). CHS and CHI are two early enzymes that drive the flavonoid pathway towards formation of naringenin, the common precursor of many flavonoid compounds including anthocyanins, flavan-4-ols and possibly 3-deoxyanthocyanidins (Dong et al., 2001; Winkel-Shirley, 2001a; 2001b). In response to fungal infection, sorghum suppresses the anthocyanin pathway to channel resources and energy for the activation of alternative pathway required for 3-deoxyanthocyanidin biosynthesis (Lo and Nicholson, 1998). To shunt naringenin towards 3-deoxyanthocyanidins, it is crucial to induce late enzymes in the pathway such as DFR, F3'H and ANS. DFR catalyzes the reduction of the carbonyl group in the C ring of narigenin and/or eriodictyol, in an NADPH-dependent reaction, to apiferol or luteoferol, respectively (Stich and Forkmann, 1988; Halbwirth et al., 2003). F3'H drives 3'-hydroxylation of the B ring of either naringenin and/or apiferol to form eriodictyol and/or luteoferol respectively. In addition, F3'H may also play a role in conversion of apigeninidin into luteolinidin (Lo et al., 1999; Wharton and Nicholson, 2000; Boddu et al., 2004). ANS has been suggested to catalyze conversion of flavan-4-ols

to their corresponding 3-deoxyanthocyanidins (Winefield et al., 2005). Interestingly, our gene expression analysis revealed that the genes encoding these enzymes are coordinately induced during the biosynthesis of 3-deoxyanthocyanidins. Here, we want to point out that many of the above enzymes are also involved in anthocyanin biosynthesis suggesting that these genes might be encoded by gene families. In fact, sorghum has at least eight CHS encoding genes with possible functional diversity as shown by the functional differences between CHS8 and the rest (Lo et al., 2002). There are two homologous *dfr* genes in the sorghum genome (Chen et al., 1997). In addition, the sorghum genome has at least four genes encoding F3'H. Two of these genes, *Sbf3'h2* and *Sbf3'h3* (accession, DQ787856 & AY675076, respectively) are induced after fungal infection while *Sbf3'h1* (accession no. DQ 787855) is activated during anthocyanin biosynthesis in response to light (Boddu et al., 2004; Shih et al., 2006). Our current results indicate that at least one copy of each of *chs*, *chi*, *dfr,* and *f3'h* is induced during biosynthesis of 3-deoxyanthocyanidin phytoalexins. Similar coordinated induction of biosynthetic genes involved in phytoalexin biosynthesis has been reported in other plant systems. For example, the biosynthesis of camalexin in Arabidopsis has been associated with transcriptional activation of the biosynthetic genes required in the camlexin biosynthetic pathway (Zhao and Last, 1996; Schuhegger et al., 2007). In rice, the biosynthesis of sakurantein was shown to be correlated with accumulation of naringenin and induction of naringenin O-methyltransferase (Kodama et al., 1992; Rakwal et al., 2000; 2001). Whether there is a common regulator of such coordinated induction of these genes in these two model plants has not yet been reported.

The activation of biosynthetic genes working in secondary metabolic pathways is often coordinated by transcription factors (Nessler, 1994; Broun et al., 2006). The light induced anthocyanin and the fungus-induced 3-deoxyanthocyanidins are under the control of different regulators (Hipskind et al., 1996a). Thus the coordinated induction of *chs*, *chi*, *dfr*, and *f3'h* reported here supports the idea of a common transcription factor. Our gene expression analysis revealed that the expression of these genes is *y1*-dependent. In addition, our investigation indicated that the accumulation of *y1* transcripts was quick (3 hpi) whereas the accumulation of the flavonoid biosynthetic genes peaked several hours post inoculation. The sequence of transcript accumulation suggests a possible regulatory role of *y1*. In fact, it has been previously shown that an R2R3 MYB transcription factor encoded by the maize *pericarp color1* (*p1*), an ortholog of *y1*, regulates the transcription of *a1*, *chs*, and *chi* (Stafford, 1990; Grotewold et al., 1994; Boddu et al., 2006). Thus in sorghum mesocotyls, *y1* may have a similar regulatory role during 3-deoxyanthocyanidin biosynthesis. The sequence $CC^{T}/_{A}ACC$ has been reported as the recognition sequence of maize P1 (Grotewold et al., 1994). Interestingly, we found a similar sequence in a number of the cloned sorghum flavonoid genes including the *Sbchs* genes (*Sbchs2, Sbchs4, Sbchs5*; accession numbers AF152549, AF152551, and AF152552, respectively), and *Sbf3'h3* (accession number AY675076). Although *Sbf3'h2* is also induced during biosynthesis of sorghum phytoalexins, we were unable to query the presence of the $CC^{T}/_{A}ACC$ motif because of the unavailability of the promoter sequence of this gene.

The early induction of *y1* upon fungal infection suggests that the initial events of fungal spore germination may generate a signal that is recognized by specific factor(s), which eventually leads to the activation of *y1* expression. Interestingly, the molecular analysis of the 5' regulatory sequences of the *y1* gene revealed that the *y1* promoter contains multiple *cis* acting elements identical to those found in many plant defense genes (F. Ibraheem and S. Chopra, unpublished results). For example, the *y1* promoter is enriched with a number of W boxes organized in a unique manner (data not shown). These boxes have been shown to be the recognition sequences of plant specific WRKY proteins which are elicitor responsive and have been implicated in regulating many plant defense responses (Rushton et al., 1996; Eulgem et al., 1999; Yang et al., 1999; Singh et al., 2002; Yoda et al., 2002; Dong et al., 2003; Xu et al., 2004). In cotton cell suspension cultures, the fungal elicitor- and methyljasmonate-induced sesquiterpene phytoalexin biosynthesis was shown to be associated with up regulation of both *GaWRKY1* and *CAD1-A* genes. *CAD1* encodes (+)-δ-cadinene synthase, a sesquiterpene cyclase that drives a branch-point reaction in the biosynthesis of sesquiterpene phytoalexins. Its promoter contains multiple W boxes and the interaction between *GaWRKY1* and these W boxes has been demonstrated (Xu et al., 2004). In addition, many WRKY proteins have been reported to work downstream of leucine rich repeat (LRR) receptor kinase (Asai et al., 2002). In sorghum, it is reported that inoculation of sorghum seedlings with *C. graminicola* induces accumulation of the SLRR-encoding cDNA (Hipskind et al., 1996b). This SLRR has high homology with the extra-cellular binding domain of the Arabidopsis receptor-protein kinases RLK5 and TMK1. Thus a possible explanation for

the early induction of *y1* transcription is that the recognition of the fungus or fungal elicitor by sorghum tissues triggers induction of at least one WRKY protein which activates transcription of *y1*. Consequently, the accumulated Y1 protein binds to the MYB recognition sequence in the promoter of its targets in the flavonoid pathway and activates their transcription. In our study, the molecular and biochemical differences between *y1-ww-1* and *Y1-rr-3* demonstrate the expected regulatory nature of *y1*. It has been hypothesized that a mutation in a regulatory gene will affect the expression of more than one gene in the pathway and eventually reduces the efficiency of the whole pathway (Forkmann, 1994; Glazebrook and Ausubel, 1994). Thus, our gene expression analysis supports our biochemical data and both suggest that the functional *y1* controls biosynthesis of sorghum 3-deoxyanthocyanidin phytoalexins.

In response to infection by the anthracnose fungus, *C. sublineolum*, sorghum plants carrying a functional *y1* allele accumulated all known 3-deoxyanthocyanidins and had a significant reduction in anthracnose disease symptoms indicated by the localized red spots on the infected leaves. On the other hand, the *y1-ww-1* line was deficient in biosynthesis of 3-deoxyanthocyanidins and exhibited symptoms typical of anthracnose disease. The antifungal activity of these 3-deoxyanthocyanidins particularly luteolinidin and methoxy luteolinidin has been well established (Nicholson et al., 1987; Snyder and Nicholson, 1990; Weiergang et al., 1996; Nielsen et al., 2004). Cytological studies have shown that these compounds distort the fungal membrane structures and restrict growth to the primary infection areas (Nicholson et al., 1987; Snyder and Nicholson, 1990; Lo et al., 1996; Wharton and Julian, 1996; Nielsen et al., 2004). Similar restriction of *Puccina*

coronata hyphal growth has reported in avenalumin I-producing oat plants (Mayama et al., 1995).

Together with induction of 3-deoxyanthocyanidins, it has been reported that fungal infection separately stimulates other defense mechanisms such as PR proteins (Lo et al., 1999). The genetic origin of *y1-ww-1* suggests that this line would be able to express all other defense mechanisms as does *Y1-rr-3*. These results highlight the potential contribution of 3-deoxyanthocyanidin phytoalexins in sorghum resistance against anthracnose disease. Our investigation also provides genetic evidence that the enhanced resistance of *Y1-rr-3* stock is due large extent to the functional *y1* gene which leads to transcriptional activation of the flavonoid pathway to produce 3-deoxyanthocyanidin phytoalexins at the right time, in the epidermal cells under attack, and in concentrations high enough to restrict or kill the pathogen. In contrast, in the *y1-ww-1* line, a complete absence of Y1 leads to an active inhibition of the pathway. The role of *y1* in controlling the biosynthesis of 3-deoxyanthocyanidin phytoalexins is in agreement with the well established role of R2R3 MYB transcription factors in driving plant specific responses (Stracke et al., 2001). For example, more than 125 R2R3 MYB encoding genes are present in Arabidopsis and a large number of these genes contribute to its defense transcriptome (Eulgem, 2005). R2R3 transcription factors have been shown to be involved in the regulation of cell death (Vailleau et al., 2002), phenylpropanoid pathway (Borevitz et al., 2000), flavonoid pathway (Grotewold et al., 1994; Boddu et al., 2005; 2006), and responses to biotic and abiotic stresses such as UV radiation, drought, salinity, and oxidative stress (Jin and Martin, 1999; Mengiste et al., 2003). The unique

expansion of R2R3 MYB transcription factors in plants suggested the presence of strong positive selection pressure for these regulators to enable plants to cope with environmental stress (Martin and Paz-Ares, 1997; Jin and Martin, 1999). Thus we believe that the picture that is emerging now is one in which different R2R3 transcription factors are linked to different defense mechanisms *in planta*.

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Figure legends

Figure 1. Schematic representation of biosynthetic pathway of anthocyanins, phlobaphenes, and the postulated branch of 3-deoxyanthocyanidin phytoalexins in sorghum. PAL deaminates phenylalanine to cinnamic acid, which undergoes aromatic hydroxylation by C4H to form p-coumaric acid. Then 4CL catalyzes the activation of *p*-coumaric acid to form *p*-coumaroyl CoA. Subsequently, CHS catalyses the stepwise condensation of one molecule of coumaryl CoA and three molecules of malonyl-CoA as the first step in the flavonoid biosynthetic pathway to form naringenin chalcone. The latter then undergoes stereospecific isomerization by CHI to form naringenin, the common precursor of anthocyanins, phlobaphenes and may be 3-deoxyanthocyanidin phytoalexins. The fate of naringenin is determined by the genetic constitution of the plant and the environmental conditions. Enzymes name are: PAL, Phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-hydroxycninnamic acid:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase, DFR, dihydroflavonol reductase; F3′H, flavonoid 3′ hydroxylase; ANS, anthocyanidin synthase. ANS was included in the pathway based on speculated role of this enzyme (Winefield et al., 2005).

Figure 2. Phenotypic and molecular structure comparison of sorghum genetic stocks carrying *Y1-cs-30*; *Y1-rr-3*, and *y1-ww-1* alleles. A. Seed pericarp and mature leaf phenotypes. B. DNA gel blot analysis of *Y1-rr-3* (R), *y1-ww-1* (W), and their common progenitor *Y1-cs-30* (C). Genomic DNA was isolated from sorghum leaves and digested independently with four restriction enzymes: *Bam*HI, *Hin*dIII, *Sal*I, and *Sca*I. The DNA digests were electrophoretically resolved, blotted and gel blots were hybridized with either intron 2-specific DNA fragment F-3 of *Y1-rr* or *y1*-cDNA. Molecular weight markers in kbp are shown between the blots.

Figure 3. Gene structure of the *y1-ww-1* allele showing an internal deletion.

A. Diagrammatic representation of the structure of *y1* in *Y1-rr-3* and *y1-ww-1*. A bent arrow shows the transcription start site. Black boxes represent exons that are joined by lines representing introns. The inverted triangle represents the *candystripe*1 (*cs1*) transposon inserted in the intron 2 of *Y1-cs* allele. The proper excision of *cs1* gives rise to the functional *Y1-rr-3* allele while improper excision of *cs1* produces the null *y1-ww-1* allele. The incomplete triangle in the map of the *y1-ww-1* represents the remnants of the transposon. The dotted line in *y1-ww-1* illustration, in comparison to *Y1-rr-3*, indicates sequence deletion. The restriction enzyme sites shown are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; SA, *Sac*I; SL, *Sal*I; SC, *Sca*I; X, *Xho*I.

B. PCR analysis of sorghum stocks with functional and null *y1* alleles. PCR reactions were carried out using genomic DNA isolated from mesocotyls from each of *Y1-cs-30*, *Y1-rr-3* and different *y1-ww* alleles as a template and two sets of primer pairs: I, YF-1 and YR-1, and II, YF-2 and YR-2 were used. The positions of these primers are shown in Fig. 3A. Lanes are: 1, *Y1-cs-30*; 2, *Y1-rr-3;* 3, *y1-ww-1*; 4, *y1-ww-4*; 5, *y1-ww-264*; 6, *y1-ww-971*; 7, negative control contains primers but no genomic DNA.

Figure 4. Comparative response of the etiolated mesocotyls of *Y1-rr-3* and *y1-ww-1* after inoculation with *Cochliobolus heterostrophus*.

A. Accumulation of reddish-brown phytoalexin compounds in *Y1-rr-3* and *y1-ww-1* mesocotyls at 0, 24, and 36 hpi (hours post-inoculation) with *C. heterostrophus* inoculation. The upper part of each picture shows the response of a representative mesocotyl carrying *Y1-rr-3* allele while the lower part shows that of a mesocotyl carrying the *y1-ww-1* allele.

B. TLC analysis of methanolic extracts prepared from inoculated *Y1-rr-3* and *y1-ww-1* mesocotyls. The methanolic extracts were loaded on a TLC plate and developed using a mixture of CH_3COOH: HCl:H_2O (30:3:10, v/v) as a solvent. Pure luteolinidin and apigeninidin were loaded as standards. Extract prepared from the sorghum line carrying the *Y1-rr-3* allele shows two bands that co-migrate with luteolinidin and apigeninidin

standards, while no bands were detected in the lane carrying extract from the *y1-ww-1* allele.

Figure 5. Spectrophotometeric confirmation of induced compounds.
A. Comparison of the absorption spectra of the acidic-methanol extracts of *Y1-rr-3* and *y1-ww-1* mesocotyls before and after infection with *C. heterostrophus* . The upper panel is for extracts prepared from *y1-ww-1* (curve 1) and *Y1-rr-3* (curve 2) at 0 hpi. The lower panel is for uninoculated and inoculated *y1-ww-1* extracts (curves 1 and 4) and uninoculated and inoculated *Y1-rr-3* extracts (curves 2 and 3) obtained 24 hpi.
B. Spectrophotometric quantification of methanolic extracts of 3-deoxyanthocyanidin phytoalexins prepared from un-inoculated and inoculated *Y1-rr-3* and *y1-ww-1* mesocotyls harvested at 0, 24, and 36 hpi. Absorbance was recorded at 480 nm. The error bars represent the standard error of three replicates and the experiment was repeated thrice independently.

Figure 6. Characterization of the 3-deoxyanthocyanidins phytoalexins in *Y1-rr-3* and *y1-ww-1* in response to *C. heterostrophus*.
A. HPLC chromatograms of methanolic extracts of 3-deoxyanthocyanidins from equivalent amount of inoculated mesocotyls of *Y1-rr-3* and *y1-ww-1* at 480 nm. Pure luteolinidin and apigeninidin were eluted at approximately 10 and 11 min respectively.
B. LC-MS analysis of methanolic extracts of 3-deoxyanthocyanidins from inoculated mesocotyls of *Y1-rr-3* and *y1-ww-1*. The LC- MS profile of the *Y1-rr-3* extract shows three major and a number of minor ions. The major ions at 269.1, 271.1, and 285.2 m/z (mass/charge ratio) which correspond to methoxy-apigeninidin (MA), luteolinidin (L), and methoxy-luteolinidin(ML), respectively. One of the minor ions at 255 m/z that corresponds to apigeninidin (A). Other minor ions could not be identified. The profile of *y1-ww-1* extract shows some trace amounts of luteolinidin and methoxy-apigeninidin.

Figure 7. Transcriptional expression analysis of *y1* and the flavonoid structural genes in inoculated sorghum *Y1-rr-3* and *y1-ww-1* mesocotyls.

A. Slot blot hybridization of radioactively labeled first strand cDNA from RNA isolated 24 hpi from sorghum mesocotyls. For each gene, 200 ng of the purified and denatured gene fragment or linear plasmid vector DNA was added per slot and transferred to nitrocellulose membrane using a slot blotter. Empty means no DNA was added as a control to monitor background hybridization.

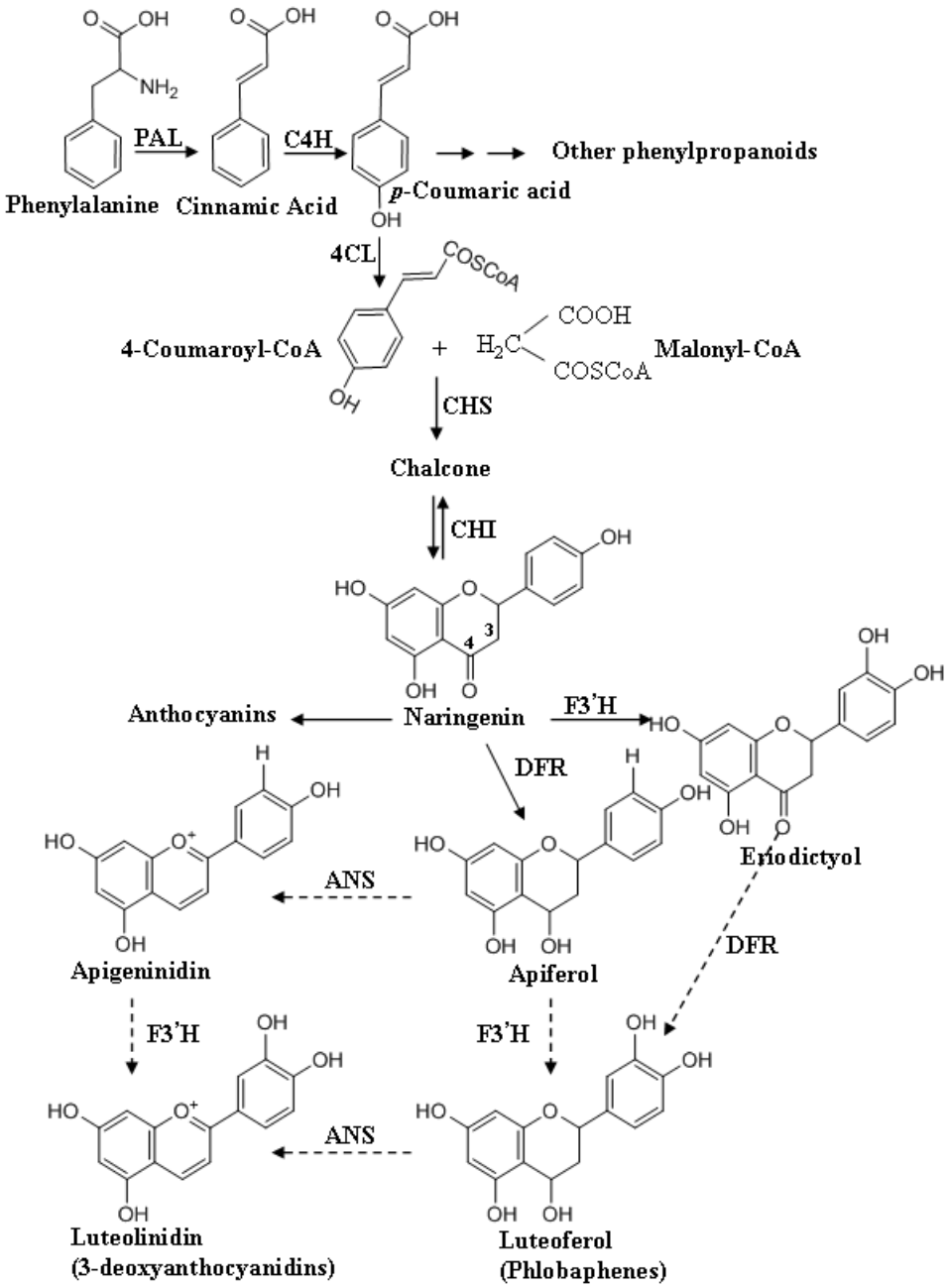
B. Temporal expression of sorghum *y1* and flavonoid structural genes in *Y1-rr-3* mesocotyls after inoculation with *C. heterostrophus*. Shown are RNA gel blots carrying 5 μg of total RNA extracted at different time intervals after inoculation and probed with cDNA clones for *yellow seed1* (*y1*), chalcone synthase (*chs*), chalcone isomerase (*chi*), flavonoid 3' hdyroxylase (*f3'h*), and dihydroflavonol reductase (*dfr*). Numbers at the top represent hours after inoculation with *C. heterostrophus*.

Figure 8. Disease symptoms and 3-deoxyanthocyanidin phytoalexin biosynthesis in *Y1-rr-3* and *y1-ww-1* plants in response to inoculation with sorghum anthracnose fungus.

A. Two-week old seedlings were challenged with C. *sublineolum* and disease symptoms were recorded two and seven days post inoculation. Inoculated leaves showing hypersensitive response-like phenotype after two days post inoculation (panel a). Inoculated leaves showed restriction of anthracnose disease seven days after inoculation in the case of *Y1-rr-3* and typical symptoms of disease in *y1-ww-1* (panel b).

B. HPLC analysis of the induced 3-deoxyanthocyanidin compounds three days after infection with *C. sublineolum.* The upper panel is a chromatogram of pigments extracted from inoculated seedlings carrying the *Y1-rr-3* allele. Chromatogram shows accumulation of luteolinidin (L, retention time 13.63 min), Apigeninidin (A, retention time 14.2 min) and 7-methoxy-apigeninidin (MA, retention time 16.2 min). The lower panel shows chromatograms of pigments extracted from inoculated plants carrying the *y1-ww-1* allele and this profile revealed the absence of most of the known 3-deoxyanthocyanidins.

Figure 1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Cinnamic Acid
p-Coumaric acid
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Malonyl-CoA
CHS
Chalcone
CHI
Anthocyanins
Naringenin
F3'H
DFR
ANS
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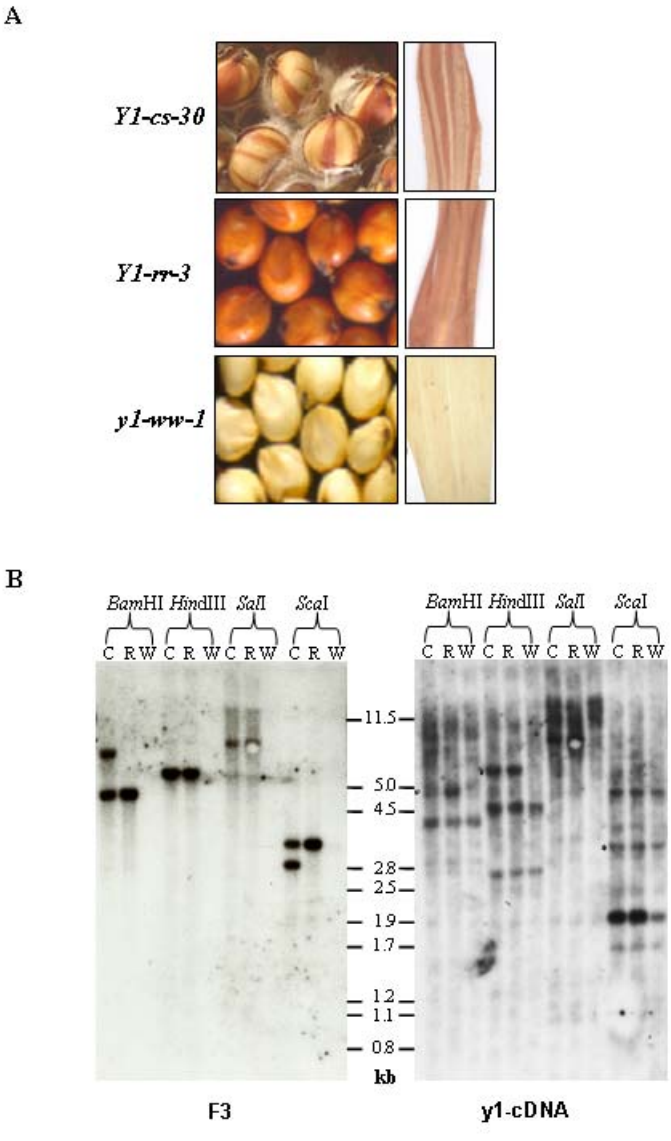
Apiferol
Luteolinidin
(3-deoxyanthocyanidins)
Luteoferol
(Phlobaphenes)

Figure 2


A
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Y1-rr-3
y1-ww-1
B
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11.5
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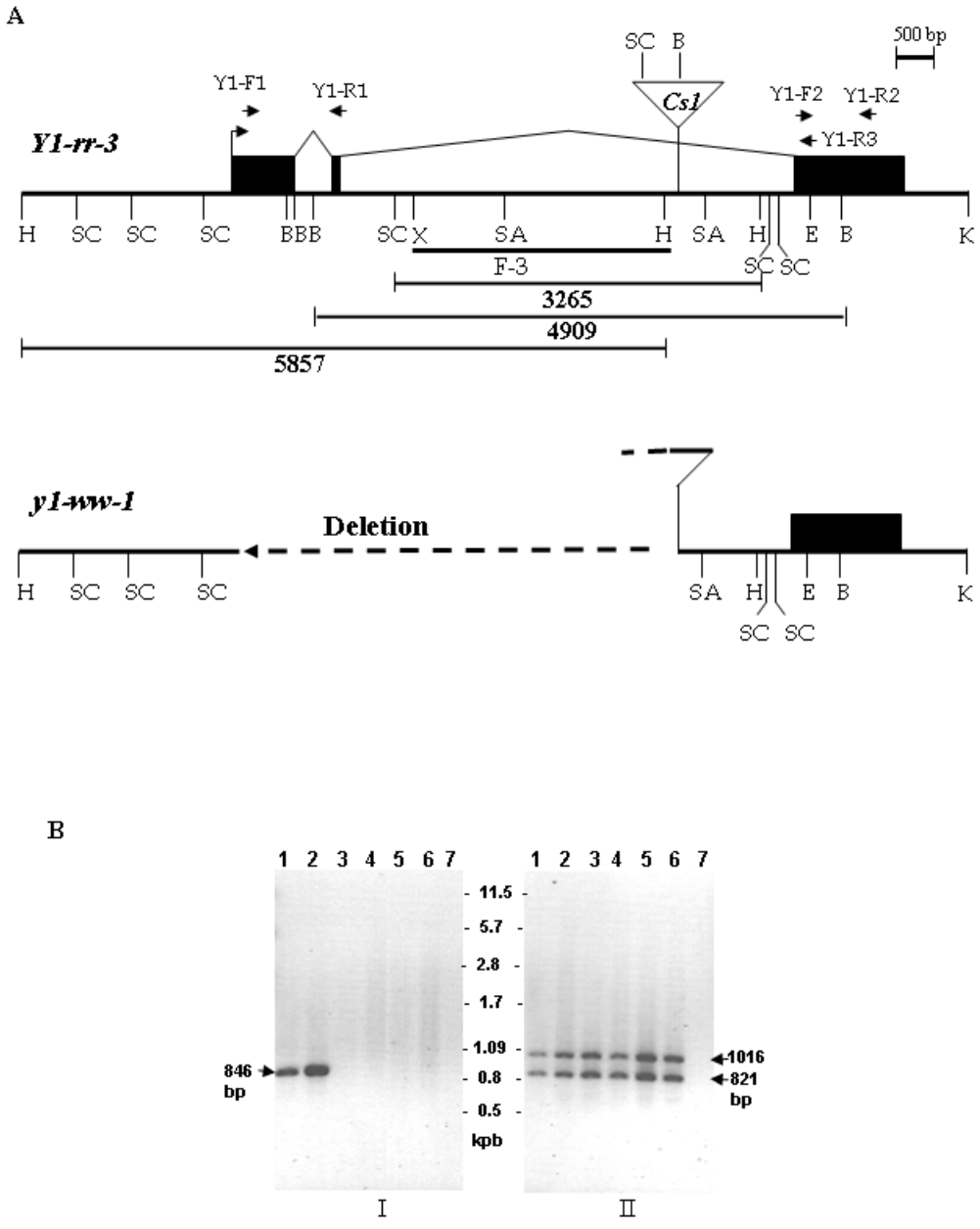
F3
y1-cDNA

Figure 3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F-3
SC SC
3265
4909
5857
y1-ww-1
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H SC SC SC SA H E B K
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1 2 3 4 5 6 7
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Figure 4

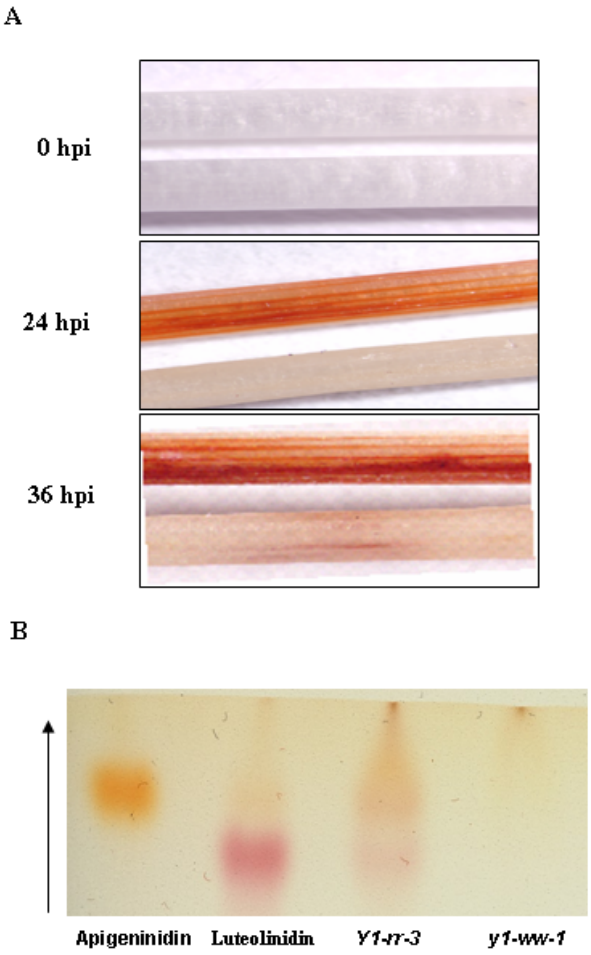
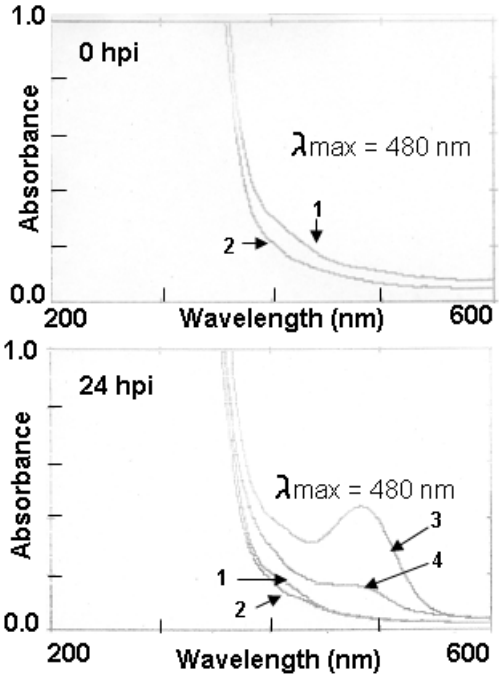

A
0 hpi
24 hpi
36 hpi
B
Apigeninidin
Luteolinidin
Y1-rr-3
y1-ww-1

Figure 5

A


1.0
0 hpi
Absorbance
λmax = 480 nm
1
2
0.0
200
Wavelength (nm)
600
1.0
24 hpi
Absorbance
λmax = 480 nm
3
4
1
2
0.0
200
Wavelength (nm)
600

B

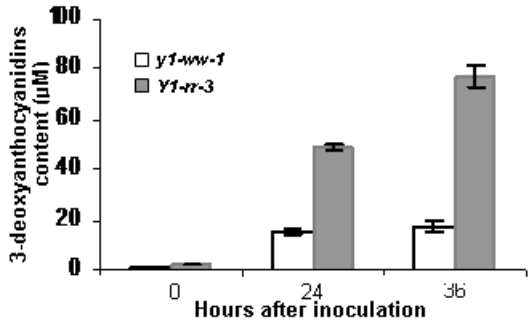
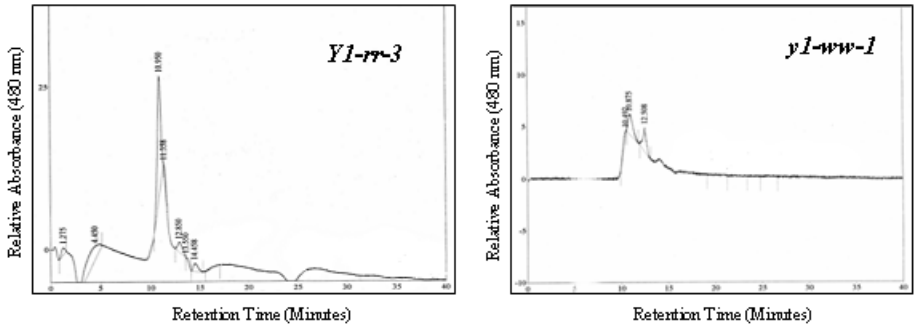

100
80
60
40
20
0
3-deoxyanthocyanidins content (uM)
y1-ww-1
Y1-rr-3
0
24
36
Hours after inoculation

Figure 6

A


Y1-rr-3
y1-ww-1
Relative Absorbance (480 nm)
Retention Time (Minutes)

B

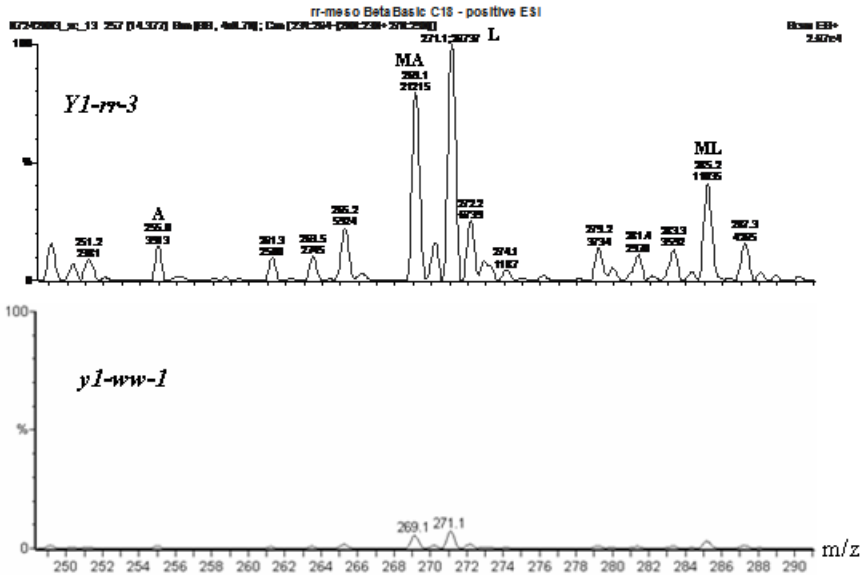

rr-meso BetaBasic C18 - positive ESI
Y1-rr-3
y1-ww-1
A
MA
L
ML
269.1
271.1
m/z

Figure 7

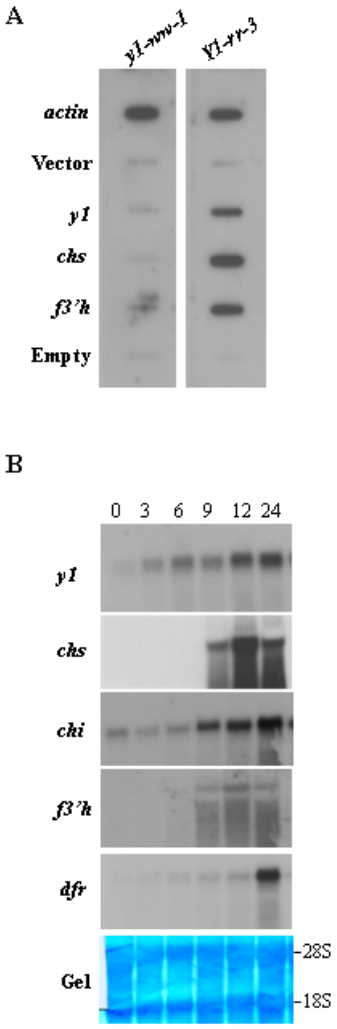
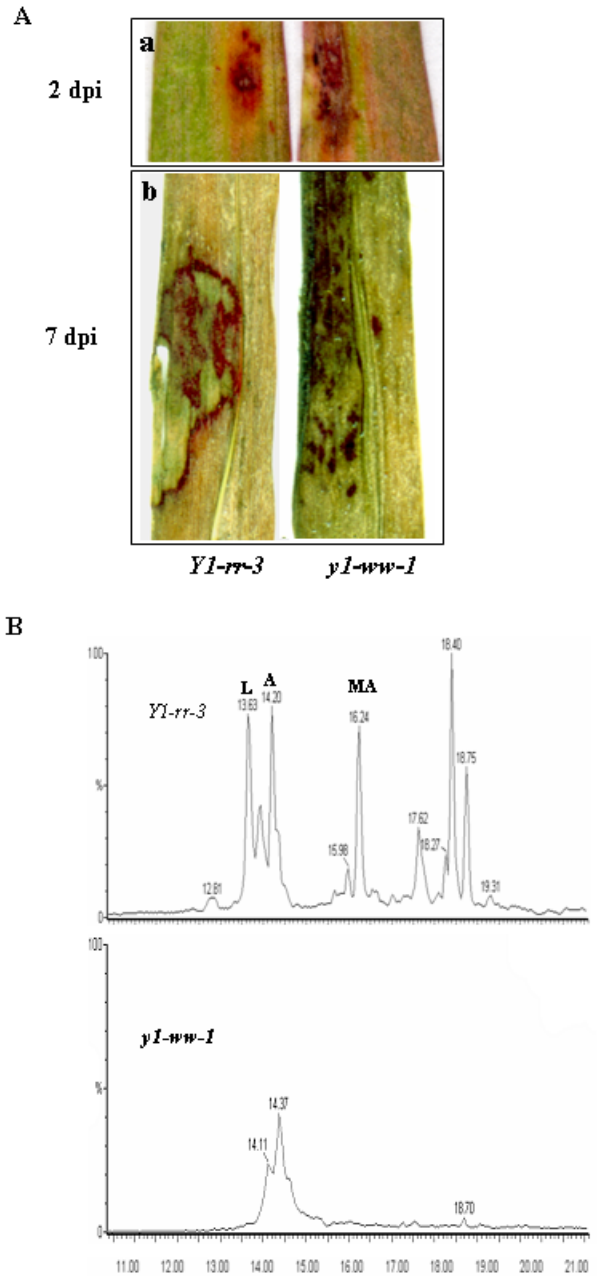

A
y1-mw-1
Y1-yv-3
actin
Vector
y1
chs
f3'h
Empty
B
0 3 6 9 12 24
y1
chs
chi
f3'h
dfr
Gel
28S
18S

Figure 8


A
a
2 dpi
b
7 dpi
Y1-rr-3
y1-ww-1
B
Y1-rr-3
L
13.63
A
14.20
MA
16.24
12.61
15.98
17.62
16.27
18.40
18.75
19.31
y1-ww-1
14.37
14.11
18.70
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00

Chapter 3

Heterologus expression of sorghum Myb transcription factor *yellow seed1* in maize

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Abstract

The significant contribution of flavonoids and phenolics to plant defense against pathogens has generated considerable interest in engineering these compounds in the economically important crops such as maize. Sorghum and maize are closely related species, with high level of co-linearity between their genomes. However, the flavonoid pathway in these two species shows some interesting differences. For example, sorghum accumulates flavan-4-ols, the precursor of the red phlobaphene pigments, in the floral tissues as well as in the leaves. In maize, these compounds are synthesized in the floral tissues only. In addition, sorghum responds to the challenges of pathogenic and nonpathogenic fungi by production of 3-deoxyanthocyanidin phytoalexins. On the other hand, maize lines that have a similar set of functional flavonoid genes do not synthesize 3-deoxyanthocyanidins in response to fungal attack. In sorghum, the biosynthesis of flavan-4-ols and 3-deoxyanthocyanidins requires a functional *yellow seed1* (*y1*) gene, an orthologe to the maize *pericarp color1* (*p1*) gene in maize. *y1* and *p1* exhibit high degree of similarity in their coding sequences but very poor homology in the regulatory regions.. These two genes have almost the same pattern of expression except that *y1* has a significant expression in leaves and is induced by fungal infection. We developed transgenic maize lines expressing either an *Y1::GUS* fusion or the intact *y1* gene and used these plants to test the expression of *y1* in maize leaves and its induction by the fungus. Our analysis indicated that the *y1* promoter has a constitutive expression in maize floral and vegetative tissues and is responsive to the fungal infection. In addition, *y1* induced pigmentation phenotypes in maize floral tissues as well as leaves and these phenotypes were stably inherited in subsequent generations. Further, *y1* drives the flavonoid pathway in maize leaves towards the biosynthesis of flavan-4-ols, and enriched the maize tissues with a variety of defense related phenylpropanoids and flavonoids. The accumulation of these defense related compounds in transgenic maize leaves was correlated to an enhanced resistance against southern corn leaf blight.

Introduction

Zea mays (corn) is one of the most economically important cereal crops of the world. In 2007, the total area planted under corn for all purposes was 92.9 million acres with about 85.4 million acres for grain production in the United States alone (US Department of Agriculture, National Agricultural Statistics Service). In the field, maize plants continually encounter biotic challenges caused by a wide variety of pathogens. Anthracnose caused by *Collteotricum spp* and leaf blight caused by *Cocholiobolus heterostrophus* are among the most serious fungal diseases that affect corn productivity. In addition, insect pests like earworm, *Helicoverpa zea*, can cause losses in corn yield by feeding on the exposed silks and developing kernels or by facilitating fungal infection. These fungal diseases and insect pests pose serious health and food safety risks especially during epidemics (Ortega et al., 1980; Bergstrom and Nicholson, 1999). For example, the epidemic of anthracnose and Southern Corn Leaf Blight (SCLB) caused a sever loss of corn yield in 1970s (Bergstrom and Nicholson, 1999). Applications of synthetic fungicides and pesticides have been used to control these infestations, but their economic cost and environmental impacts have been of concern for both producers and consumers. To prevent further epidemics and reduce the need for synthetic chemicals, there is an ongoing search for maize germplasm with natural resistance against these diseases. Engineering this trait using genes from other plant species has also been one of the priorities of the national program.

In maize, the flavonoid pathway gives rise to many defense related compounds such as flavan-4-ols and C-glycosyl flavones. Flavan-4-ols are the precursor of the brick

red phlobaphene pigment that accumulates in mature pericarp and cob tissues (Fig. 1) (Styles et al., 1987; Stafford, 1990; Grotewold et al., 1994). Their biosynthesis requires a functional *pericarp color1* (*p1*), a gene encoding an R2R3 myb transcription factor (Styles and Ceska, 1989; Grotewold et al., 1994). *p1* also controls about 60% of the trait for maysin biosynthesis in the silk (Byrne et al., 1996; Grotewold et al., 1998; Zhang et al., 2003; Cocciolone et al., 2005). Maysin is a C-glycosyl flavone that provides natural resistance against insects (Waiss et al., 1979; Snook et al., 1994). Its insecticidal activity is attributed to disruption of the amino acid metabolism in the insect gut (Byrne et al., 1996). Interestingly, the resistance against insects, maysin content, and pericarp pigmentation phenotypes are all correlated to the silk browning phenotype, a monogenic trait in which the silk cuttings attain brown color a few minutes after damage (Levings and Stuber, 1971; Byrne et al., 1989; Byrne et al., 1996; Cocciolone et al., 2005).

We have begun comparative characterization of the flavonoid pathways in sorghum and maize (Boddu et al., 2006). These two species are genetically related and have been suggested to have diverged from a common ancestor more than 16.5 million years ago (Bennetzen and Freeling, 1997; Gaut et al., 2000). The two genomes have a high degree of synteny and sequence similarity (Melake-Berhan et al., 1993; Chen et al., 1997; Devos and Gale, 2000). The co-linearity between these two species suggests a similarity between their metabolic pathways. In fact, sorghum has been shown to accumulate phlobaphene pigment in the pericarp. It is well documented that the biosynthesis of these pigments is under the control of *yellow seed1*, an orthologe of maize *pericarp color1* (Zanta et al., 1994; Chopra et al., 1999; Boddu et al., 2006). It has been

reported that *y1* and *p1* activate the transcription of chalocone synthease (*chs*), chalcone isomerase (*chi*), and dihydroflavonol reductase (*dfr*) during biosynthesis of flavan-4-ols in sorghum and maize respectively (Grotewold et al., 1994; Chopra et al., 2002; Boddu et al., 2006). In addition, the mesocotyls of these two species accumulate similar profiles of anthocyanins in response to light (Hipskind et al., 1996).

Regardless of the above similarities, the flavonoid pathways in sorghum and maize exhibit a number of interesting differences. For example, in maize, phlobaphene pigments are obvious in the floral tissues, husk and leaf sheath but not in leaf blade. However in sorghum, phlobaphene pigments appear in floral tissues as well as in the mature leaf blade. These patterns of phlobaphene accumulation are in a complete agreement with *p1* and *y1* patterns of expression in the two species (Boddu et al., 2006). Another interesting difference is the response of these two species to fungal attack. Sorghum responds to anthracnose and leaf blight fungi by the induction of red-brown phytoalexins belonging to the 3-deoxyanthocyanidins class (Snyder and Nicholson, 1990). However, maize lines carrying a similar set of functional flavonoid regulatory and structural genes do not synthesize any 3-deoxyanthocyanidins in response to *C. heterostrophus* infection (Hipskind et al., 1996). With the exclusion of *chs* in maize, neither the flavonoid structural nor regulatory genes showed induction after fungal infection (Hipskind et al., 1996). Interestingly, silks of some maize lines have been reported to constitutively accumulate very low levels of luteolinidin (McMullen et al., 2001). However, the significance of the low levels of these compounds in defense against fungi and other microbes has not been tested. To the best of our knowledge, neither

luteolinidin nor any of the other 3-deoxyanthocyanidins have been reported in maize leaf in response to fungal attack.

Sorghum 3-deoxyanthocyanidin phytoalexins include apigeninidin, luteolinidin and their derivatives. Upon fungal challenge, these compounds accumulate around the primary infection sites and prevent further proliferation of fungus within sorghum tissues (Nicholson et al., 1987; Snyder and Nicholson, 1990; Lo et al., 1999). These compounds have been shown to inhibit fungal germ tube growth and distort fungal structures (Wharton and Julian, 1996; Lo et al., 1999).Their potent antifungal activity against *C. sublineolum*, *C. graminicola*, and *C. hetrostrophus* has been demonstrated (Nicholson et al., 1987; Snyder and Nicholson, 1990; Aguero et al., 2002). Interestingly, Sorghum 3-deoxyanthocyanidins have a structure similar to flavan-4-ols, and their biosynthesis involves up regulation of the expression of *chs*, *chi*, *dfr*, and *f3'h* . The induction of these genes requires functional *yellow seed1* (*y1*) (Boddu et al., 2005). Interestingly, the *y1* mutant was deficient in 3-deoxyanthocyanidins and exhibited typical symptoms of anthracnose susceptibility in comparison to the resistant wild type (Chapter 2).

y1 and *p1* have been cloned (AF209212 & AY860968) and the analysis of their genomic sequences indicated a high level of similarity (92%) at their coding sequence but very poor homology in the non coding regions (Grotewold et al., 1994; Boddu et al., 2006). These findings gave us an insight to possible evolutionary modifications that might have happened in *p1* and *y1* and account for the functional diversity of the flavonoid pathway in these two closely related species. Therefore, the main objective of the current paper was to test the hypothesis that "in sorghum and maize, the spatial

difference in phlobaphene accumulation and induced 3-deoxyanthocyanidin biosynthesis is a property of the *y1* promoter".

To test this hypothesis, we developed transgenic maize plants that express different *y1* constructs and used these lines to investigate the heterologous expression of sorghum *y1* in maize. Our results demonstrated that the *y1* transgenes are active in maize tissues and are responsive to fungal infection. In addition, our biochemical analysis established that *y1* successfully drives the maize flavonoid pathway towards production of flavan-4-ols, maysin, and other defense related compounds in maize leaves and other tissues.

Material and methods

Plasmid construction

All plasmids used in this study were developed based on the pBluescript II vector (Stratagene, La Jolla, CA). The plasmid p*Y1::GUS* contains the 5' regulatory region of sorghum *y1* gene (AY680968, Fig. 2A), *Escherichia coli* β –glucuronidase (*gus A*) reporter gene (Jefferson et al., 1987) and potato *PinII* gene terminator sequence (An et al., 1989). This plasmid was constructed by ligating a 2375 bp *Hin*dIII-*Bam*HI promoter fragment of *y1* to the *Bam*HI-*Eco*RI fragment carrying *GUS* and *PinII* sequences (Cocciolone et al., 2000). This cassette was cloned into *Hin*dIII-*Eco*RI digested pBluescript II vector (Fig. 2B). The plasmid p*Y1::Y1* contains a 9164 bp *y1* genomic fragment containing 2 kbp of the 5' regulatory region, 6946 bp sequence including three exons and two introns, and an 820 bp sequence downstream of 3'UTR. This plasmid was

prepared by inserting the *Hin*dIII-*Kpn*I DNA fragment of *Y1-rr* gene into *Hin*dIII-*Eco*RI digested pBluescript II vector (Fig. 2C).

Cell culture and transient expression assay

Black Mexican Sweet (BMS) maize cell suspension cultures were used as a target tissue for the transient expression assay. These cells have the *p1-ww* (null *p1* allele), *A1 A2 C1 C2 R-g b pl* genotype (Grotewold et al., 1998). BMS cell cultures were maintained on solid and/or liquid MS media (Murashige and Skoog, 1962) supplemented with vitamin (M5519, Sigma), 30 mg L^{-1} sucrose, and 1.5 mg l^{-1} dichlorophenoxy acetic acid(2, 4-D). To test the heterologous activity of 5' regulatory sequence of the *y1* in maize tissue, BMS cells were transformed with the *pY1::GUS* plasmid and the transient activity of GUS was monitored as explained below. For transformation, the BMS cells were subcultured in a fresh liquid media and were incubated at 28°C on a shaker at 150 rpm. Seventy two hours after subculture, the BMS cells were treated with 0.2 M manitol, an osmotic stressor, for 4 h and then cells were plated onto fresh solid media. In the mean time, the *pY1::GUS* plasmid DNA ($1\mu g\mu l^{-1}$) was precipitated onto 1.1 μm tungsten particles in the presence of $CaCl_2$ and spermidine (Klein et al., 1988). Aliquots of tungsten suspension were loaded onto a macrocarrier and bombarded into the BMS cells using PDS 1000/HE biolistic gun (BIORAD, Hercules, CA). Conditions of bombardment include 1100 psi rupture disc, 9 cm target distance, and 28 Torr vacuum. Each plate was bombarded twice and then incubated at 26 °C in dark for 48 h. The bombarded cells were scraped from plates and processed for qualitative GUS assay (see below).

Tissue culture, transformation, and regeneration of transgenic maize plants

Immature zygotic embryos derived from *Hi II* maize line (Armstrong and Green, 1985) were used to develop friable embryogenic type II calli. Callus induction, maintenance, and transformation were carried out according to a previously described protocol (Frame et al., 2000). The stable transformations were carried out in the transformation facility at Iowa State University. A total of 20 *Y1::GUS* and 16 *Y1::Y1* independent transgenic events were generated from herbicide resistant calli. The selection for transgenic plants in T_1 and subsequent generations was based on bialphos (AgrEvo, Wilimington, DE) resistance and PCR analyses (Fig. 3). The Bialphos application procedure and PCR details are available upon request. The transgenic plants were maintained in a hemizygous state by out-crossing with pollen from the inbred line 4Co63 which carries a *p1-ww* allele (*p1* non-expressing line). Progeny derived from such cross always segregated in 1:1 ratio indicating stable expression patterns of the transgenic plants included in this study. All maize plants carrying either p*Y1::GUS* or p*Y1::Y1* transgenes exhibited wild type growth and morphology when compared with non transgenic maize plants.

Histochemical and fluorometric analysis of GUS activity

GUS histochemical assays were performed as previously described (Jefferson et al., 1987). Cells and tissues were immersed in a stain solution containing the chromogenic substrate 5-bromo-4- chloro-3-indolyl-B-D-glucuronide (X- gluc, Sigma,

St. Louis) and incubated at 37 °C for 24 h. Tissues were then cleared by several washes with 70% ethanol. Stained cells and tissues were photographed using a dissection SMZ1000 microscope attached to a DXM1200F digital camera (Nikon, NY, USA).

For quantification of GUS activity, a known fresh weight of leaf tissues were ground under liquid nitrogen and homogenized in a solution containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM dithiothreitol, 1 mM EDTA, 0.1% sodium lauryl sarcosine, and 0.15 % Triton X 100. The homogenate was centrifuged at 14000 rpm for 5 min at 4 °C and the supernatant (enzyme extract) was collected in fresh tubes. GUS activity was assayed by incubation of a known volume of enzyme extract with assay buffer (50 mM sodium phosphate pH 7.0, 10 mM dithiothreitol, 1mM EDTA, 0.1% sodium lauryl sarcosine, 0.15 % Triton X 100, and 1 mM 4-methylumbelliferyl β-D-glucuronide (MUG)) at 37 °C for 1 h. The enzymatic reaction was stopped by addition of 0.2 M sodium carbonate and fluorescence was measured using TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, CA). The protein content of the plant extract was measured using protein dye-binding assays (Bradford, 1976).

Induction of *y1* in transgenic maize plants by fungal attack

Induction of the *y1* promoter upon fungal infection was tested by inoculating maize plants with *Cochliobolus heterostrophus* and studying the response by detached leaf assay (Coca et al., 2004). *C. heterostrophus* was used because of the rapid induction of *y1* in sorghum and the resulting accumulation of phytoalexins in the infected tissues (Lo and Nicholson, 1998; Aguero et al., 2002). Furthermore, this fungus is the causal

agent of southern leaf blight of maize. *C. heterostrophus* was grown on potato dextrose agar (PDA) medium under continuous light at room temperature for 10 days. Conidia from the fungal colonies were collected in sterilized water containing 0.001% Tween 20. At 15 days after pollination (dap), leaf discs from both sides of the midrib of L6 (the 2nd leaf above the 1st ear) leaves were harvested. Discs on the left side of the midrib were used as controls. Discs were then placed, with the abaxial surface facing upward, on water agar (1% w/v) supplemented with 2 mg/l kinetin. Whatman filter papers soaked in a spore suspension of *C. heterostrophus* (10^5 spores /ml) were placed on the leaf discs. Plates were incubated either in dark (for *Y1::GUS*) or under illumination (for *Y1::Y1*) at 28°C. The filter papers were removed after 24 h and plates were kept under the same conditions until harvest.

Image analysis for evaluation of disease response

The disease phenotypes on the *Y1::Y1* transgenic plants and control genotypes were recorded using a dissection microscope (Nikon SMZ1000) connected to Nikon digital camera (DXM1200F). For quantitative analysis, images were processed by Automated Lesion Extraction using algorithms (VphenoDBS) developed for a visual phenotype database (Shyu et al., 2007). This technique depends on differentiating the lesion pixels (foreground) from the normal ones (background) and measuring the area of lesions. The percentage of the infected area was used to evaluate disease severity.

Determination of Total phenolics and flavanoids

L6 leaves from greenhouse-grown plants were collected at the time of pollination. Leaves were frozen in liquid nitrogen and subsequently freeze dried. Twenty milgrams of ground tissue was washed three times in ether to remove waxes and chlorophyll pigments, and extracted three times under sonication in 70% acetone supplemented with 1 mM ascorbic acid. The supernatant was collected and acetone was evaporated using a speed vacuum drier and the remaining extract was used for determination of total phenolics and total flavonoids. For mesurement of total phenolics, 0.075 ml of Folin-Ciocalteu reagent was added to 0.075 ml of the plant extract followed by incubation for 6 min at room temperature. Subsequently, 0.075 ml of 2N sodium carbonate solution was added followed by incubation at room temperature for 1 h. The intensity of the developed color was recorded at 760 nm using SpectraMAX 190 plate reader (Molecular Devices Crop., Sunnyvate, CA). Phenolic content was expressed as mg gallic acid equivalents g^{-1} dry tissue (Appel et al., 2001; Wolfe et al., 2003).

The flavonoid content in plant extracts was measured according to Wolf et al. (2003) with minor modifications. Briefly, 250 µl of plant extract was mixed with 75 µl of 5% sodium nitrite and the mixture was incubated at room temperature for 5 min. Subsequently, 150 µl of 10 % $AlCl_3$ solution was added followed by another incubation for 6 min at room temperature. Finally, 500 ul of 1 M NaOH was added and absorbance was recorded at 510 nm using SpectraMAX 190 plate reader (Molecular Devices Crop., Sunnyvate, CA). Total flavonoid content was expressed as mg catechin equivalent g^{-1} dry tissue (Wolfe et al., 2003).

Detection and determination of flavan-4-ols

To detect the presence of flavan-4-ols, 30 mg of ground leaf tissue was washed in ether and suspended in 500 µl of 30 % HCl/70 % butanol mixture. The homogenate was incubated at 37°C for 1 h followed by centrifugation at 14000 rpm for 10 min. The supernatant was screened over a wavelength range from 450 to 650 nm using an UV mini-1240 spectrophotometer (Shimadzu Scientific Instruments, Inc. Columbia, MD). For quantitation of flavan-4-ols, the concentration of relative flavylium ions was measured by recording the absorption at 550 nm (Grotewold et al., 1998).

HPLC analysis of phenylpropanoids, maysin, and other flavonoids

Silk samples were collected from covered primary ears three days after silk emergence and immediately frozen in liquid nitrogen. Leaf samples were collected from mature L6 leaves of green house-grown plants at 20 dap. The silk and leaf samples were freeze dried and used for analysis of maysin, isoorinetin, and chlorogenic acid. One hundred mg of freeze dried tissue was extracted in absolute methanol at 0°C for 14 days. The resulting extracts were warmed to 26°C, mixed with chrysin (internal standard), sonicated for 20 min, and equal aliquots of the extracts were analyzed using reversed-phase HPLC. The program of analysis included water/methanol linear gradient from 20 % to 80 % methanol for 35 min with a flow rate of 1ml min^{-1} using Altex Ultrasphere C18 column (4.6 X 250 mm, Beckman Instruments, Norcross, GA). C-gylcosyl flavones and chlorogenic acid were measured at 340 nm as described previously (Snook et al., 1993; Grotewold et al., 1998).

Determination of Lignin

Lignin for whole L6 leaves was measured using thioglycolate method (TGA) as described previously (Bruce and West, 1989; Brinkmann et al., 2002). Absorbance of the thioglycolate derivatives was recorded at 280 nm using SpectraMAX 190 plate reader (Molecular Devices Crop., Sunnyvate, CA). Lignin content was expressed as mg lignin TGA derivative g^{-1} dry tissue.

Results

***y1* promoter exhibits constitutive activity in maize tissues**

To test the functionality of the *y1* promoter in maize, BMS cell suspension was bombarded with *pY1::GUS* plasmid. The GUS expression analysis indicated that the *y1* promoter is functional in BMS maize cells (Fig. 4). Subsequntly, stable transgenic maize plants were developed using the same construct and different tissues of *Y1-GUS* transgenic plants were assayed for GUS activity. GUS activity was observed in husk, pericarp, cob, silk, and tassel glumes (Fig. 5A). Thus, the expression pattern specified by the *y1* promoter in the floral tissue was similar to that of the endogenous *P1-rr* allele. Interestingly, GUS activity was also observed in the anthers, embryos, and leaves - tissues in which the endogenous *P1-rr* does not show any appreciable activity. In leaf, the GUS expression was not uniformly distributed which may be due to the uneven diffusion of X-gluc through mature maize leaf (Cocciolone et al., 2000).

To record the activity of the *y1* promoter during plant development, we looked at GUS expression in different leaves of transgenic plants. Our results indicated that the *y1* promoter was functional in all leaves but the level of expression showed an interesting trend. The GUS activity was highest in the first leaf (near the bottom of the plant), showed sharp decline in the second leaf, and progressively decreased in the subsequent leaves towards the top of the plant (Fig. 5B).

Sorghum *y1* is induced by fungal infection in maize tissues

To investigate the possible activation of the *y1* promoter by fungal attack, we used *Y1::GUS* maize plants as a reporter system. The transgenic plants were challenged with C. *heterostrophus* and the GUS activity was monitored using histochemical and fluorometric assayes. As expected, the uninoculated leaves showed scattered, faint blue spots indicating a low level of activity of the *y1* promoter (Fig. 6A-a & c). On the other hand, presence of highly localized, intensive blue spots on inoculated leaves indicated induction of the *y1* promoter by the fungus (Fig. 6A-b & d). Quantification of the GUS protein activity indicated that the *y1* promoter is constitutively active (Fig. 6B). However, fungal inoculation resulted in a significant up regulation of GUS activity reflecting induction of *y1* by fungal ingress. Further, we also observed significant increase in the GUS activity over time after inoculation, indicating sustained activity of the *y1* promoter upon fungal attack. In the control plants, we observed small fluctuations in the GUS activity throughout the experiment. These changes could be attributed to wounding or

physiological alterations that might have occured in the leaf under the experimental conditions (Nooden and Guiamet, 1996).

Transformation with *y1* gene induces different pigmentation phenotypes in maize

The activity of the sorghum *y1* promoter in maize tissues and its induction by fungal infection prompted us to develop transgenic maize lines expressing the intact *y1* gene and study the induction of phlobaphene pigments in various plant tissues. Remarkably, the *y1* transgene induced three distinct patterns of pericarp pigmentation in three transgenic maize lines arising from independent transformation events. These patterns included uniformly red, patterned with a red dot at silk attachment point, and colorless (white) pericarp (Fig. 7A). The cob glumes in these lines were red, pink, and dark red, respectively. Based on the pericarp and cob glume pigmentation patterns of Y1-maize plants, and the system of nomenclature used for endogenous *p1* alleles, we named these maize transgenic events as *Y1-rr* (red pericarp, red cob); *Y1-pr* (patterned pericarp, red cob); and *Y1-wr* (white pericarp, red cob). Sibling maize plants lacking the *y1* transgene exhibited white pericarp and white cob glumes and were designated as NS (negative segregants). In addition, *y1* also induced accumulation of phlobaphene pigments in husk and tassel (Fig. 7B and C). Apart from the red pigmentation observed in these tissues, an orange color was observed in mid-rib. The mid-rib pigmentation appeared at early stages of growth (3 leaf stages) and lasted till the time of maturity (Fig. 6D). Additionally, the silk tissue of *y1*-mazie plants showed browning upon wounding, a phenotype that was not observed in the NS plants (Fig 7E). The silk browning phenotype,

also observed in the plants carrying endogenous *p1* alleles, has been correlated with *p1*-induced phlobaphene pigmentation (Cocciolone et al., 2005). However, stronger browning phenotype in Y1-maize plants indicated that the transgene was more effective in producing this phenotype. The distinct phenotypes produced by *Y1-rr*, *Y1-wr*, and *Y1-pr* were stably inherited across generations.

y1-induces biosynthesis of 3-deoxyflavonoids in the floral and vegetative tissues of transgenic maize

The red phlobaphene pigmentation specified by *p1* and *y1* in maize and sorghum, respectively, is resulted from polymerization of flavan-4-ols. These compounds are also considered as precursors of 3-deoxyanthocyanidins phytoalexins (Stich and Forkmann, 1988; Styles and Ceska, 1989; Schutt and Netzly, 1991; Grotewold et al., 1998; Wharton and Nicholson, 2000; Chopra et al., 2002; Winefield et al., 2005). To confirm that pigmentation observed in the transgenic plants was due to accumulation of phlobaphenes, and to compare the levels of these compounds in different genotypes, we performed biochemical analysis. Spectral analysis of acid-butanolic extracts from pericarp, cob and tassel glumes, anthers, mid-rib, and leaf showed accumulation of flavan-4-ols in the transgenic plants (Fig. 8A&B). Detection of these compounds in leaf tissues was quite surprising as we did not observe visible pigmentation. Quantification of flavan-4-ols in leaf tissue showed that Y1-maize transgenic plants had significantly higher levels of these compounds as compared to the endogenous *p1* alleles. However, variable levels of flavan-4-ols were observed among different transgenic events with *Y1-pr* having the

highest and *Y1-wr* the lowest accumulation (Fig. 9). These results indicated that *y1* is more effective in driving flavonoid pathway in leaf toward production of flavan-4-ols.

y1 induces accumulation of phenolics and flavonoids in maize

To investigate the effect of *y1* on other derivatives of phenylpropanoid pathway, we looked at the accumulation of phenolics and flavonoids in transgeneic plants. Quantitative measurement of these compounds in leaf showed significantly higher accumulation in *Y1-pr* and *Y1-rr* as compared to *Y1-wr* and the endogenous *p1* alleles (Fig. 10A and B). The high level of total phenolics and flavonoids could either be due to increased accumulation of compounds already present in maize plants carrying endogenous *p1* alleles, or due to appearance of novel metabolites. To address this question, reverse-phase HPLC was used to compare the phenolic and flavonoid profiles in leaves of different genotypes. The genotypes carrying endogenous *p1* allele and negative segregants had similar profiles which comprised of approximately 12 peaks (Fig. 11A and C). Interestingly, transgenic *Y1-rr* maize showed greater variety of phenolics and flavonoids as indicated by detection of 24 peaks (Fig. 11B). While half of the peaks were common between transgenic and endogenous *p1* carrying plants, approximately 12 unique peaks were observed in *Y1-rr* plants. Of these, four peaks (retention time 9.46, 12.14, 12.98, 15.22 min) most likely represent soluble glucosides or ester derivatives of caffeic or ferulic acid. Three peaks with retention time 22.19 and 23.747, and 24.008 min corresponded to maysin, apimaysin, and methoxymaysin, respectively. Finally, identity of other novel peaks present in *Y1-rr* was unknown. In

summary, *y1* triggered the biosynthesis of known as well as some novel phenolic and flavonoid compounds in maize.

y1-induces biosynthesis of maysin in silk and leaf tissues

Detection of maysin and related C-glycosyl flavones in the HPLC profile of transgenic plants prompted us to investigate the impact of *y1* on biosynthesis of these compounds in maize. In most of previous studies, maysin has been shown to accumulate in silk tissue but presence of these compounds in leaf is not well documented (Casati and Walbot, 2005). Comparison of silk and leaf tissue showed that maysin content was generally higher in silk (Fig. 12A& B). In silk, *Y1-pr* and *Y1-rr* had significantly higher levels of maysin as compared to the two endogenous *p1* alleles. Interestingly, the impact of *y1* on maysin accumulation in leaves was more dramatic; *Y1-pr* and *Y1-rr* had 6-9 folds higher maysin as compared to endogenous *p1* alleles. Isoorintin and rhamnosyl isoorintin, two C-glycosyl flavones, showed similar patterns of accumulation in silks and leaves of the tested genotypes (Fig. 13).

y1-induces chlorogenic acid in transgenic maize

To test the possible induction of other defense-related phenylpropanoids by the *y1*, we analyzed the levels of chlorogenic acid in silk and leaf using reverse phase HPLC. Our results indicated that the content of chlorogenic acid in silks was about 4 fold higher than that in leaves across all genotypes. Further, silk derived from *Y1-rr*, *Y1-pr*, and *Y1-wr* plants had significantly higher levels of chlorogenic acid than *p1* alleles (Fig. 14A).

Cholorogenic acid content in leave was more variable across the tested genotypes; while one transgenic event (*Y1-rr*) produced significantly higher levels, the two other events did not show appreciable increase as compared to the endogenous *p1* alleles (Fig. 14B).

_y1_ and _p1_ have similar effects on lignin content in maize leaves.

In addition to producing phlobaphenes and other flavonoids, the phenylplpronoid pathway is also a source of lignin biosynthesis. Further, the orange midrib phenotype exhibited by *Y1-rr* and *Y1-pr* plants resembles that of maize brown midrib mutant which shows altered lignin composition (Halpin et al., 1998; Vermerris and Boon, 2001). To test if *y1* induced alternation of lignin content, we measured the lignin in the whole leaf using the thioglycolic acid method. The data revealed that the *y1* transgene or *p1* natural alleles did not show any dramatic differences in the lignin content (Fig. 15). However, plants carrying a functional *y1* or *p1* allele had higher levels of lignin as compared to that present in negative segregants.

Y1-transgenic maize plants exhibit enhanced resistance to _C. heterostrophus_

The *y1* expressing plants were analyzed for their performance against southern leaf blight. Because disease resistance is always a complex trait, we compared the response of *y1* transgenic to the negative segregant. In addition, we also included maize lines expressing *P1-wr* (B73) allele because it was one of the parents used to develop *HiII* line, the transgene recipient in our experiment (Armstrong and Green, 1985). Disease development on *y1* transgenics and other control genotypes was investigated

using a detached leaf assay and *C. heterostrophus*. The evaluation of disease severity on leaves was based mainly on measuring the percentage of the diseased areas using VphenoDBS software (Shyu et al., 2007). Our results indicated that the inoculated leaves of transgenic plants had a reduced number of chlorotic lesions compared to the control genotypes (Fig. 16A). These infection lesions were spread over about 30% and 62% of the leaf area in negative segregants and *P1-wr*, respectively. In contrast, the mean values of the infection area ranged from 4% to 16% among different *y1* transgenic events (Fig. 16B). These results indicate that *y1* transgenic plants had a significant reduction in disease severity compared to the control genotypes.

Discussion

Transferring desirable traits such as disease resistance to crop plants has been the ultimate goal of geneticists since the domestication of plants for human use. Disease resistance-related genes often play a direct or indirect role in protecting plants against biotic challenges. Therefore, genes that confer natural resistance against pathogens are good candidates for engineering disease resistance in economically important crops (Punja, 2001). This approach overcomes the inability of crosses between different wide species (species barrier) confronted by conventional breeding. In addition, it enables researchers to focus on a single gene or a limited number of known genes and have a precise control over where and how these gene(s) are expressed (Essenberg, 2001). It is also preferable over the use of synthetic pesticides which are neither fully effective nor environmentally safe. The expression of defense related genes in the new host system is

usually performed using either their native, pathogen-inducible, or heterologus promoters (Gurr and Rushton, 2005). Recently, we have demonstrated that a myb transcription factor encoded by *yellow seed1* (*y1*) controls the biosynthesis of flavan-4-ols and 3-deoxyanthocyanidins phytoalexins in sorghum during the plant's development or in response to fungal infection (Boddu et al., 2006). Sorghum *y1* mutants are more susceptible to anthracnose disease compared to the wild type plants (chapter 2).

In the current study, the activity of the sorghum *y1* promoter was tested using *Y1::GUS* fusion or the *y1* gene in transgenic maize lines. The functional analysis of the *y1* promoter by transient assay and stable transformation demonstrated that the *y1* promoter is active in maize tissues including leaves. These results indicated that the sequence upstream of the *y1* transcription start site contains the *cis* regulatory elements sufficient to drive a considerable level of GUS expression in the floral and vegetative tissues of maize.

In sorghum, the *y1* promoter is induced by pathogenic and non-pathogenic fungi (Chapter 2). In transgenic maize, we found that the *y1* promoter is responsive to infection with *C. hetrostrophus* and its activity increased overtime. These results indicated that the 5' regulatory sequence of *y1* used to drive GUS expression contains the *cis* regulatory sequences necessary to recognize the fungal-induced signal(s). Interestingly, the molecular analysis of this regulatory region revealed the presence of multiple *cis* elements identical to those found in many plant defense genes (Rushton and Somssich, 1998; Eulgem, 2005). For example, this sequence contains TTCGACC, an elicitor recognition element identical to that identified in the promoter of *PR2* in tobacco (Shah

and Klessig, 1996). In addition, this region is enriched with a number of W boxes organized in a specific manner (see chapter 4). These boxes have been shown to be the recognition sequences of plant specific WRKY proteins which are elicitor responsive and have been implicated in regulating many plant defense responses (Rushton et al., 1996; Eulgem et al., 1999; Yang et al., 1999; Singh et al., 2002; Yoda et al., 2002; Dong et al., 2003; Xu et al., 2004).

Transformation of *Hi II* maize line with the intact *y1* induced the accumulation of flavan-4-ols and phobaphenes in the floral as well as many vegetative tissues. These results are in agreement with the structural similarity of the Y1 and P1 which implies that the two proteins are able to bind the same target motifs thereby activating the same genes. Interestingly, the phlobaphene pigments accumulated in the floral tissues of Y1-maize plants at the exact timing of their appearance in maize and sorghum plants expressing natural *p1* and *y1* alleles, respectively. These findings suggest that, at least in these tissues, *y1* expression is developmentally regulated and the mechanism of such regulation does not depend on the transgene's position in maize genome. In addition, the homogeneity of the pigmentation (i.e., absence of variegation) suggests that the pattern of pigmentation in these plants is established before organ formation. The presence of three distinct phlobaphene pigmentation phenotypes (*Y1-rr*, *Y1-pr*, and *Y1-wr*) in the floral tissues of three independent events and the stable heritability of these phenotypes indicate that *y1* regulatory regions might have undergone an epigenetic modification in the first generation. The possible contribution of the position of the transgene in the genome to regulation of such differential pigmentation pattern cannot be ruled out.

y1 also induced synthesis of flavan-4-ols in maize leaves, a property that has not been reported for maize lines expressing either *p1*-natural alleles or *p1* transgenes (Chopra et al., 1996; Chopra et al., 2003 2005). The presence of flavan-4-ols in these leaves suggest that *y1* behaves in maize as it does in sorghum and actively interacts with the *chs*, *chi*, and *dfr* promoters to drive the falvonoid pathway towards the production of these compounds. In fact, crossing of transgenic maize lines expressing *A1::GUS* fusion with Y1-maize plants caused intensive induction of GUS activity (data not shown). In spite of the accumulation of flavan-4-ols in transgenic maize leaves, we could not detect either phlobaphenes or 3-deoxyanthocyanidins in the bleached or mature maize leaves. The absence of the phlobaphene pigments in these leaves suggests that the polymerization of flavan-4-ols to phlobaphenes in sorghum leaves occurs during physiological maturity and requires factor(s) that might be missing in maize leaves. Similar results were also obtained in BMS cells bombarded with *p1* gene. These cells accumulated luteoferol but neither phobaphenes nor 3-deoxyanthocyanidins were observed (Grotewold et al., 1998).

The biochemical analysis of the transgenic maize leaves revealed that *y1* induced significant accumulation of flavan-4-ols, flavonoids, phenolics, and the natural insecticide maysin to levels which are not commonly found in maize leaves. In general, HPLC analysis of flavonoid and phenolics in leaves of *y1-transgenic* maize has demonstrated that *y1* induces the accumulation of compounds that are indigenous to the *Hi II* background. These results are in agreement with the known regulatory role of *y1*. However, we also observed several peaks that are unique to the methanolic extracts from

leaves of *y1*-transgenic maize. Altogether, these results indicate that *y1* interacts with its expected as well as novel targets in maize.

The induction of a high level of maysin and its related C-glycosyl flavones in *y1*-maize leaves is quiet interesting. The biosynthesis of maysin in maize silk starts with naringenin and occurs under the control of *p1* and *p2* (Zhang et al., 2000). CHS is one of the early enzymes required for maysin biosynthesis (Szalma et al., 2002). It drives the stepwise condensation of malonyl-CoA with *p*-coumaroyl-CoA as the first committed step in the flavonoid pathway towards naringenin formation. Narigenin then undergoes several modifications such as 3' hydroxylation, 2.3- desaturation and 6- glycosylation of the flavonoid nucleus. Therefore, to shunt naringenin towards C-glycosyl flavones, it is crucial to induce enzymes such as F3'H, FNS as well as those necessary for glycosylation of the flavonoid A ring. The sugar modifications that shape the final structure of isoorientin and maysin have been shown to occur under the control of *sm1* and *sm2* genes respectively (McMullen et al., 2004). In the current study, the pronounced accumulation of maysin in leaves under normal growth conditions can be attributed to the activity of the *y1* gene. The accumulation of maysin in transgenic leaves demonstrates that all nodes of the biochemical machinery necessary for maysin biosynthesis are active in presence of *y1* gene. Thus it is possible that *y1* triggers the C-glyocosyl favone biosynthetic pathway by activating the genes encoding F3'H and/or FNS. In fact, the close examination of the promoter sequence of maize f3'h revealed the presence of 3 sequences similar to the P1 binding sequence identified in *a1* promoter (Grotewold et al., 1994). In addition, maize genetic stock that has active *f3'h* and the *y1* ortholog, *p1*, has been shown to accumulate

maysin in silk while mutant stock that has a null *f3'h* is defective in this trait (M. Sharma and S. Chopra, unpublished results).

y1- induced flavonoid accumulation was also correlated with accumulation of chlorogenic acid (CGA) and other phenylpropanoids that are not directly associated with the flavonoid pathway in maize silk and leaves. Interestingly, induction of the biosynthesis of these compounds in maize cell suspensions after transformation with *p1* transgene has also been reported (Grotewold et al., 1998; Bruce et al., 2000; Zhang et al., 2003). These results suggest that *p1* and *y1* transgenes play regulatory roles in the phenylpropanpoid pathway. However, the basis of this mechanism is not yet clear. One possible mechanism is that the R2R3 myb protein products of *p1* and *y1* might interact directly with genes working in the phenylpropanpoid pathway to secure the efficient flow of intermediates between the phenylpropanoid and flavonoid pathways. In fact, the *p1* transgene induced expression of *pal1* gene, which controls the flow of the amino acid phenylalanine into the phenylpropanoid pathway, in BMS maize cells (Bruce et al., 2000). In addition, it has been reported that accumulation of chlorogenic acid in some maize genotypes requires a functional *p1* gene and an additional locus called *qtl1* (Bushman et al., 2002). These authors suggested that *p1* activates the phenylpropanoid pathway to accumulate the intermediates which are then shunted towards CGA biosynthesis by the action of the *qtl1* locus. This dichotomous regulatory role of Myb transcription factors has also been demonstrated in other model systems. For example, two Myb proteins, Myb305 and Myb340, have been demonstrated to directly interact with phenylpropanpoid and flavonoid structural genes in flowers of *Antirrhinum majus*

(Moyano et al., 1996). Since *y1* and *p1* are known to activate flavonoid biosynthesis downstream of the phenypropanoid pathway, it is also possible that these transcription factors might interfere with the feed back regulation controlling the activity of enzymes working in the phenylpropanoid biosynthesis (Grotewold et al., 1998; Bushman et al., 2002). Evidence for the presence of feedback loops regulating the phenylpropanoid pathway has been reported using the exogenous application of phenylpropanoid intermediates and/or transformation with sense or antisense cDNAs encoding phenypropanoid pathway enzymes (Blount et al., 2000; Dixon et al., 2002). It is important to point out that the lignin content does not show pronounced changes in the leaves of transgenic maize which might indicate that the flavonoid pathway gives rise to a wide variety of flavonoids and thus creating a strong sink for phenylpropanoid intermediates. However, it is still unclear whether *y1* induces any alterations in the structural composition of lignin polymer in *y1*-transgenic maize.

In spite of our findings that flavan-4-ols are synthesized constitutively in leaves of Y1-transgenic maize, we were not able to find detectable levels of 3-deoxyanthocyanidins in these leaves. Flavan-4-ols have been suggested as potential precursors of 3-deoxyanthocyanidins by many authors (Kambal and Bate-Smith, 1976; Stich and Forkmann, 1988; Styles and Ceska, 1989; Schutt and Netzly, 1991; Grotewold et al., 1998; Wharton and Nicholson, 2000; Chopra et al., 2002; Winefield et al., 2005). The absence of the constitutive expression of 3-deoxyanthocyanidins in leaves might suggest that a candidate gene (s) working down stream of flavan-4-ols or eriodictyol is not effectively targeted by *y1*. Another possibility is that the biosynthesis of 3-

deoxyanthocyanidins in leaves requires additional factor (s) that are present only during active plant-pathogen interaction. In fact, sorghum mesocotyls and leaves do not accumulate 3-deoxyanthocyanidins in the absence of fungal infection.

The *y1*- transgenic plants exhibited enhanced resistance against corn southern leaf blight infection. The improved resistance of these plants is not very surprising because the *y1*-induced flavan-4-ols, flavonoids and phenolics are well known to contribute significantly to plant defense by inhibiting pathogen development and thus slowing down the disease spread. The antimicrobial activities of these compounds against a wide array of pathogens have been demonstrated (Hammerschmidt, 2005). For example, sorghum plants with high levels of flavan-4-ols in their grains and leaves exhibited better resistance against mold compared to those deficient in flavan-4-ols (Jambunathan et al., 1986; Jambunathan et al., 1990; Menkir et al., 1996). Flavan-4-ols include two main compounds, luteoferol and apiferol. Luteoferol has been demonstrated to have direct, strong, and broad biocidal effect against many fungi and bacteria. The antifungal activity of luteoferol has been demonstrated against *Alternaria brassicicola*, *A. solani*, *Botrytis cinerea*, *Colletotricum lagenarium*, *C. graminicola*, *Fusarium culmorum*, *Phytophthora infestans*, and *Pyricularia oryzae*. (Spinelli et al., 2005). In addition, its antibacterial activity against *Erwinia amylovora*, *Pantoea agglomerans*, and *Pseudomonas fluorescens* has also been reported (Spinelli et al., 2005). The growth of many of these microbes was significantly inhibited by luteoferol concentrations as low as 10 μM. Interestingly, the prohexadion-calcium-induced resistance of pome fruit trees against fire blight disease caused by *Erwinia amylovora* has been attributed to a number of novel flavonoid

compounds particularly luteoferol in the treated tissues (Stich et al., 2002; Halbwirth et al., 2003; Spinelli et al., 2005). These authors reported that prohexadion-calcium inhibits the anthocyanin biosynthetic pathway favoring the biosynthesis of defense related compounds such as eryidictyol, luteoferol, 3-deoxycatechin, and luteoliflavan (Halbwirth et al., 2003). A very similar observation has been reported in sorghum during the sorghum-fungus interaction (Lo and Nicholson, 1998). Unlike luteoferol, apiferol has been indirectly implicated in plant resistance against fungal diseases. This compound does not have direct antimicrobial activity against fungi but has been suggested to be the precursor of the fungal growth inhibitor apigeninidin (Schutt and Netzly, 1991). The antimicrobial activity of flavan-4-ols might justify their presence in the epidermal cells of the floral tissues such as pericarp and silk and in vegetative tissues such as husk and leaves. In fact, a mechanism describing the release of flavan-4-ols from their intercellular compartments to the sites of pathogen infection, similar to that of sorghum 3-deoxyanthocyanidin phytoalexins, has been proposed (Nicholson et al., 1987; Snyder and Nicholson, 1990; Spinelli et al., 2005). The strong antimicrobial activities of luteoferol and other flavonoid and phenolic compounds suggest that the steady state levels of these compounds in *y1-transgenic* maize leaves might provide a wide range and durable resistance against many fungal, bacterial, and pest diseases.

The presence of the *y1* induced compounds is in agreement with the general defense mechanisms in maize. It has been reported that the phenylpropanoid pathway is one of the metabolic pathways that are induced at early stages of maize-fungus interaction. The complexity of the metabolic pathways through which the above

mentioned natural defense related compounds are synthesized potentiates the importance of *y1* as a regulator of their biosynthesis. In addition, exploiting *y1* as a member of the R2R3 regulatory genes is in keeping with the trend of using R2R3 transcription factors to drive plant "specific responses" under stress conditions (Jin and Martin, 1999; Borevitz et al., 2000; Stracke et al., 2001; Mengiste et al., 2003; Boddu et al., 2005; Boddu et al.). Thus characterization of Y1-transgenic maize-microbe interaction and the molecular dissection of the *y1*promoter will be very important. This information could then be applied to other economically important crops. Such results will add to the general belief that exploitation of defense related R2R3 myb transcription factors is a promising strategy to improve plant defense mechanisms in plants.

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Figure legends

Figure 1. Schematic representation of the biosynthetic pathways of phenylpropanoid and flavonoid compounds discussed in the text. Enzymes names are: PAL, Phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:coenzymeA ligase; CHS, chalcone synthase; CHI, chalcone isomerase, DFR, dihydroflavonol reductase; F3′ H, flavonoid 3′ hydroxylase; FNS, Flavone synthase ; CGT, C-glycosyl transferase. Modeled after Zhang et al. (2003); Grotewold et al. (1998); and McMullen et al. (2004).

Figure 2. Diagrammatic representation of the transgenic cassettes.

A. Structural features of the *y1* gene. The gray box represents the upstream regulatory, while a bent arrow shows the transcription start site. Black boxes correspond to exons that are joined by bent lines representing introns. The restriction enzyme sites shown are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; SA, *Sac*I; SL, *Sal*I; SC, *Sca*I; X, *Xho*I. Illustration is drawn to scale

B. *Y1*::GUS construct used for transient expression assay and stable transformation of BMS cell line and *Hi II* maize. The promoter region shown in *y1* structure (A) was fused with the GUS reporter gene (open box). The patterned box represents the *PinII* gene terminator sequence. The illustration is not drawn to scale. The restriction enzyme sites shown are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I.

C. *Y1::Y1* construct used for stable transformation of *Hi II*. This plasmid was prepared by inserting the *Hin*dIII-*Kpn*I DNA fragment of *Y1-rr* gene (A) into *Hin*dIII-*Eco*RI sites of pBluescript II vector.

Figure 3. Screening of transgenic maize plants.

A. Basta painting assay. Transgenic plants harboring the *bar* gene are Basta resistant (a). On the other hand, maize plants segregating for the absence of the transgene exhibited necrosis on leaf surface within three to five days after herbicide application and are Basta susceptible (b).

B. PCR analysis of Y1-transgenic maize. PCR reactions was performed using leaf genomic DNA and *y1* specific primers. Lanes are: M, DNA molecular weight marker; c, *Y1::Y1* plasmid; 2, 4, 14 are Basta sensitive plants;17, negative control; and the rest lanes are Basta resistant plants.

Figure 4. Transient expression analysis of *Y1::*GUS construct in maize BMS cells. After bombardment, GUS activity was tested using GUS qualitative assay using (X- gluc) as a substrate. Shown are two independent transformation events.

Figure 5. Characterization of the expression of *y1* promoter in transgenic maize.

A. Histochemical GUS staining in different tissues of transgenic maize plants. Tissues are young leaf (a), old leaf (b), wounded young leaf (c), tassel glumes (d), kernel (e), pealed pericarp (f), embryo (g), mature anther (h). husk (i), and silk (j).

B. Quantitative analysis of GUS activity in leaves of transgenic maize plants. Activity was measured using GUS fluorometric assay and 4-methylumbelliferyl β-D-glucuronide as a substrate. Activity measured as μM of 4 MU/mg protein/min (Y- axis) was

normalized to sibling plants segregating for absence of the *Y1::GUS* transgene. Shown are the mean values ± standered error of GUS activity.. X- AXIS depicts leaf location from L1 to L9 (see materials and methods).

Figure 6. *y1* promoter is responsive to fungal challenge.

A. Histochemical analysis of *Y1::*GUS activity in detached leaves of transgenic maize plants in response to infection with *Cochliobolus heterostrophus*. Panel a shows an uninoculated while panel b shows inoculated leaf.

B. Quantitative analysis of the induction of *Y1*::GUS in leaves of transgenic maize. Detached leaf assay was carried out and the level of GUS activity was measured at different time intervals using fluorometric GUS assay in the presence of MUG as a substrate. Shown are mean values ± standard error.

Figure 7. *y1* induces different pigmentation phenotypes in transgenic maize.

Three *y1* transgenic lines were characterized: (A) pericarp and cob glumes; (B) internal husk leaf; (C) tassel; (D) Mid-rib; and (E) ear shoot showing silk cuttings. Comparable organs from maize plants segregating for the absence of *y1* transgene as well as native *p1* expressing lines were included for comparison. Names of *y1* and *p1* lines are shown below the figure.

Figure 8. Comparison of the spectral analysis of Acidic-Butanol extracts of floral and vegetative tissues from transgenic and non transgenic maize plants.

A. Spectral analysis of Acidic-Butanol extracts from sibling maize plants segregating for either the absence (a) or the presence (b) of *y1* transgene. The absorption maxima at 564 nm were eliminated by boiling confirming the presence of flavan-4-ols (c). Curves are for extracts prepared from different tissues including: Pericarp (1), Cob glumes (2), Tassel glumes (3), Anthers (4), and Midrib (5). *P-rr* Pericarp extract (6) was used as a positive control for flavan-4-ol.

B. Spectral analysis of Acidic-Butanol extracts from mature leaves of transgenic and non transgenic maize plants. Extracts from leaves of standard maize lines expressing native *P1-rr* and *P1-wr* alleles were included for comparison. Leaves were deprived of chlorophyll and Acidic-Butanol assay was performed. The clear extracts were photographed (a) and screened for the presence of flavan-4-ols (b). Curves are for leaf extracts from different plants including: 1, *Y1-wr; 2, Y1-rr; 3, Y1-pr; 4, P1-rr* pericarp (positive control for flavan-4-ols); 5, *y1-ww* (negative segregant); 6, *Hi II*; 7*, P1-rr*; and 8, *P1-wr.*

Figure 9. Flavan-4-ols in transgenic and non transgenic maize leaves.

Total flavan-4-ols were measured at 550 nm (Y1-axis). X-axis shows independent transgenic events of *Y1::Y1* and control maize plants carrying different *p1* alleles. NS represents “negative segregants” for *Y1::Y1* transgene.

Figure 10. *y1* induces accumulation of phenolics and flavonoids in transgenic maize leaves.

A. Total phenolics were expressed as mg equivalent gallic acid g^{-1} dry tissue.

B. Flavonoids content of transgenic maize plants. Flavonids content was expressed as mg catechin g^{-1} dry tissue.

Shown are the mean values ± SE. X-axis depicts Y1::Y1 transgenic events as well as maize lines carrying endogenous *p1* alleles. NS indicates negative segregants.

Figure 11. HPLC analysis of phenlypropanoids and flavonoids in transgenic maize leaves.

Shown are chromatograms for the methanolic extracts of leaves from three maize genotypes: A, negative segregant for Y1::Y1 transgene; B, Y1:Y1 (Y1-rr) transgenic line; and C, maize line with endogenous P1-rr allele. Major peaks showing absorption at 340 nm are labeled as: a, chlorogenic acid; b, caffeoyl ester; c, rhamnosyl-isoorientin; d, maysin; and 'is', the internal standard chrysin.

Figure 12. Accumulation of maysin in silk (A) and leaves (B) of transgenic and non transgenic maize plants.

Maysin content was measured using HPLC and expressed as % dry tissue weight. Different genotypes are shown on X-axis.

Figure 13. Accumulation of isoorientin and rhamnosyl isoorientin in silk (C) and leaves (D) of transgenic and non transgenic maize plants. C-glycosyl flavones were measured using HPLC and expressed as % tissue dry weight.

Figure 14. Accumulation of chlorogenic acid in silk (A) and leaves (B) of transgenic and non transgenic maize plants.

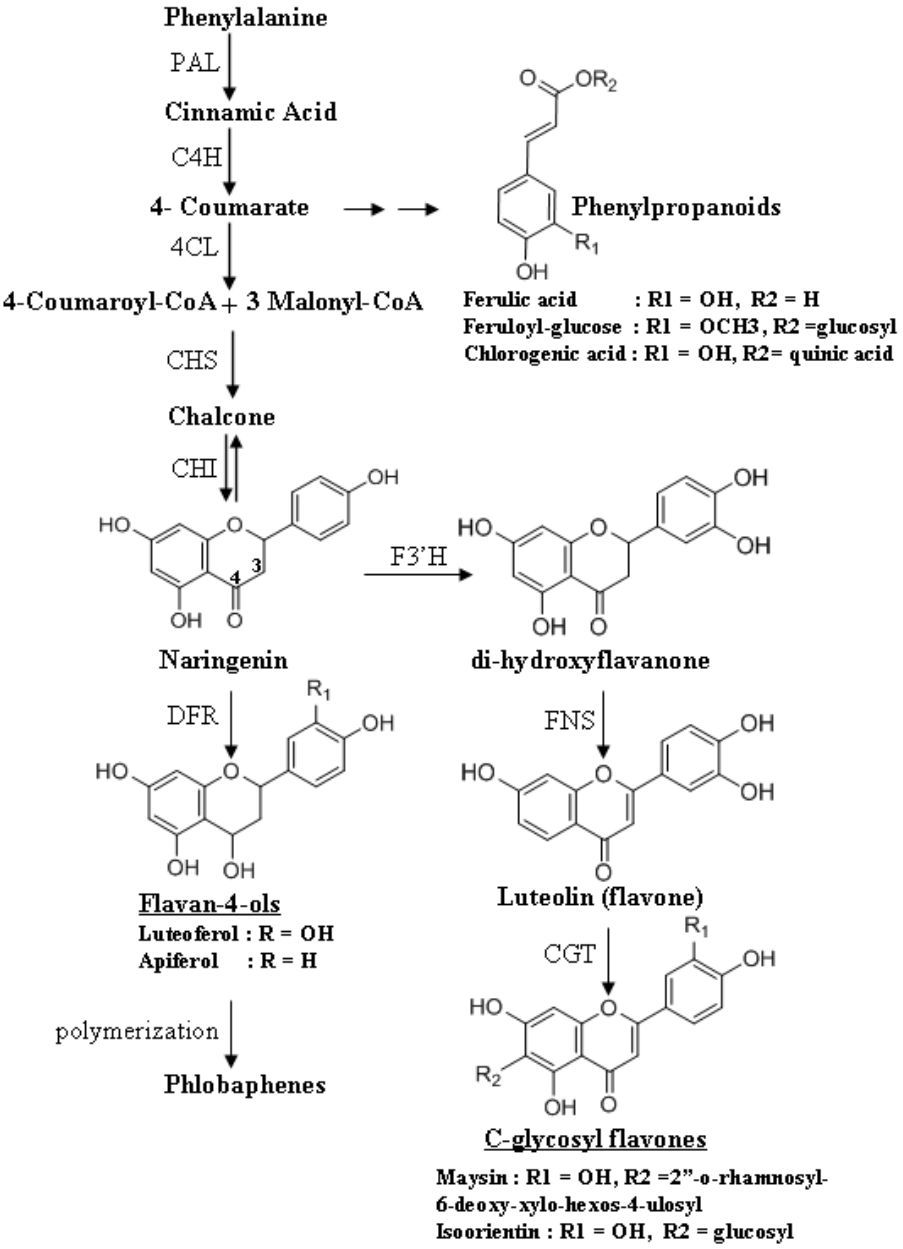
Chlorogenic acid content was measured using HPLC and expressed as % tissue dry weight. Different genotypes are shown on X-axis.

Figure 15. Lignin content in transgenic and non transgenic maize. Lignin content in leaves of transgenic and non transgenic plants was analyzed by drivatization with thioglycolic acid and the level of lignin thioglycolate derivatives were determined spectrophotometrically at 280 nm and expressed as mg g^{-1}dry tissue. Shown are the mean values ± SE.

Figure 16. *y1* enhances resistance against southern leaf blight in maize.

Detached leaves from Y1-maize plants were challenged with *Cochliobolus heterostrophus* and the disease development was monitored at 4 dpi (A). Images were recorded and processed for quantification of disease severity using VphenoDBS software (B). Shown are the mean values ± SE. UN is an uninoculated leaf while NS is a negative segregant. Other genotypes used are *Y1::Y1* transgenic maize (*Y1-pr*, *Y1-rr*, and *Y1-wr*) and endogenous p1-wr allele used as a control.

Figure 1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4CL
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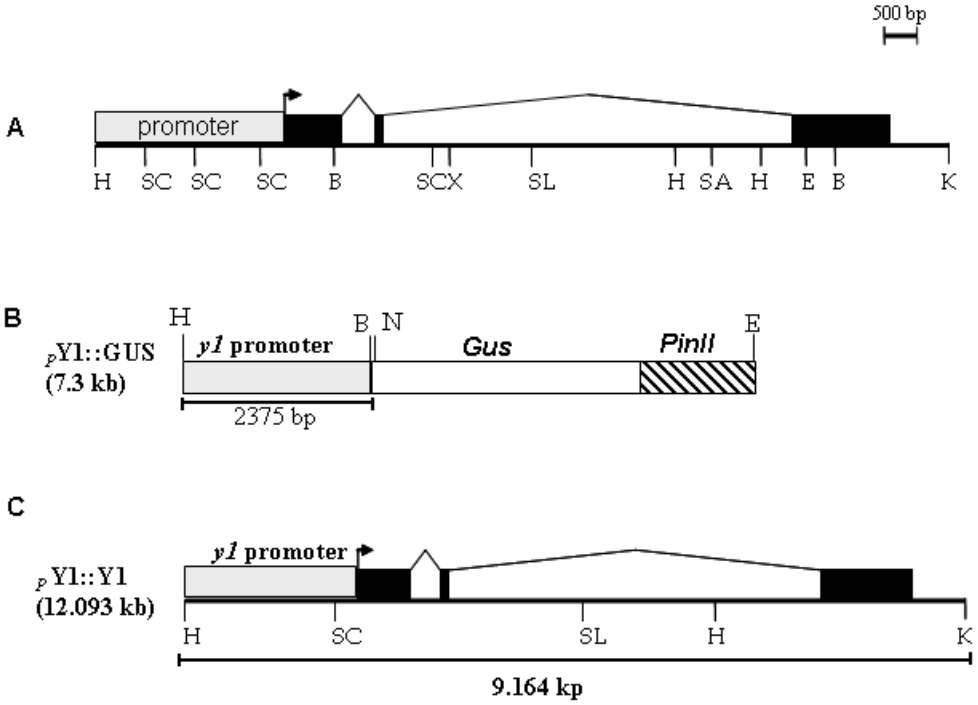
CHS
Chalcone
CHI
Naringenin
F3'H
di-hydroxyflavanone
DFR
Flavan-4-ols
Luteoferol : R = OH
Apiferol : R = H
polymerization
Phlobaphenes
FNS
Luteolin (flavone)
CGT
C-glycosyl flavones
Maysin : R1 = OH, R2 =2"-o-rhamnosyl-6-deoxy-xylo-hexos-4-ulosyl
Isoorientin : R1 = OH, R2 = glucosyl
Phenylpropanoids
Ferulic acid : R1 = OH, R2 = H
Feruloyl-glucose : R1 = OCH3, R2 =glucosyl
Chlorogenic acid : R1 = OH, R2= quinic acid

Figure 2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promoter
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H B N E
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9.164 kp

Figure 3

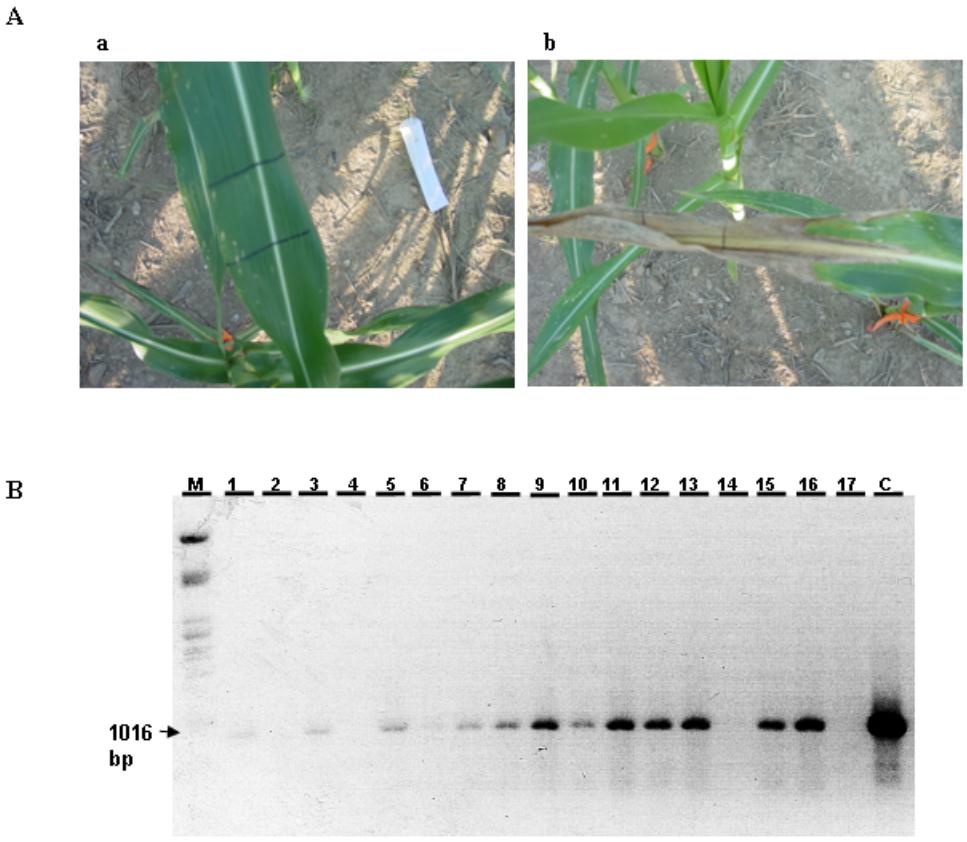
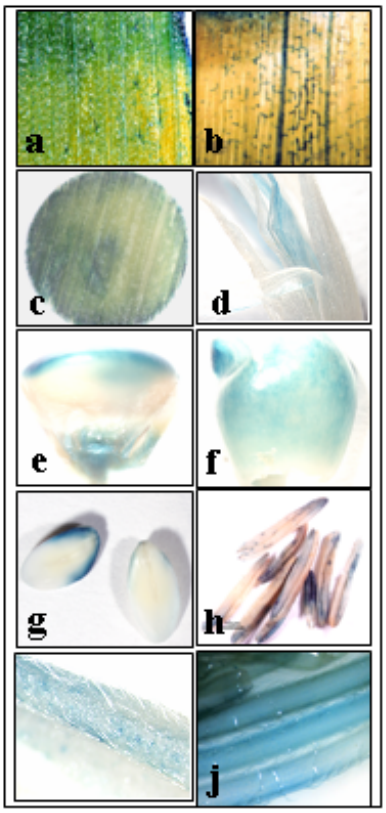


Figure 4

Figure 5

A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b
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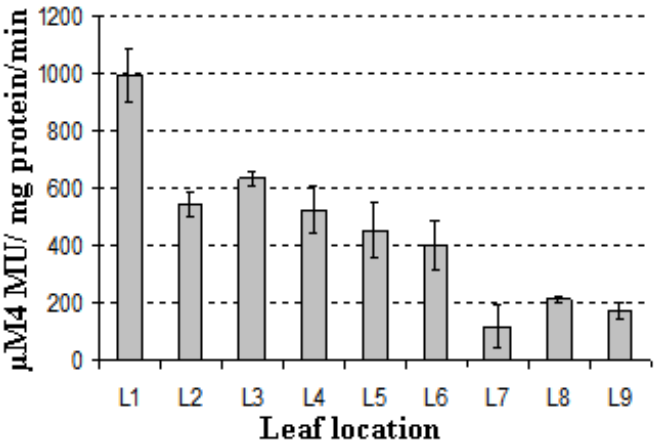
j

B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1000
800
600
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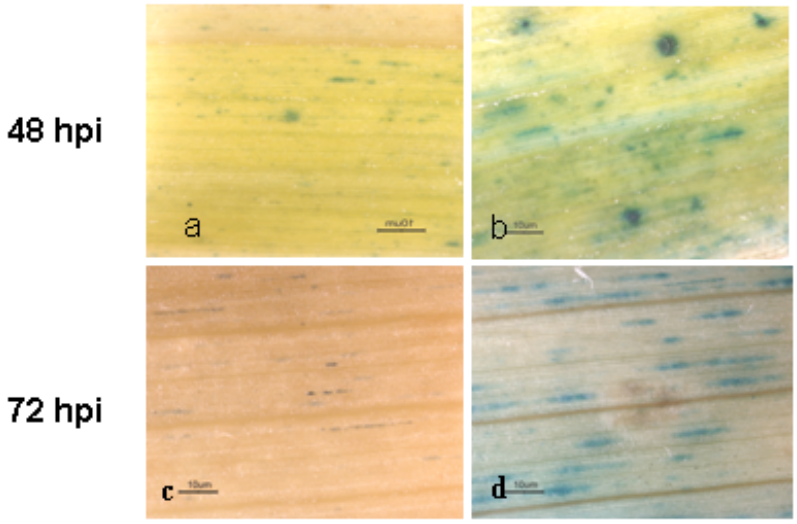
L1
L2
L3
L4
L5
L6
L7
L8
L9
Leaf location

Figure 6

A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72 hpi
a
b
c
d

B

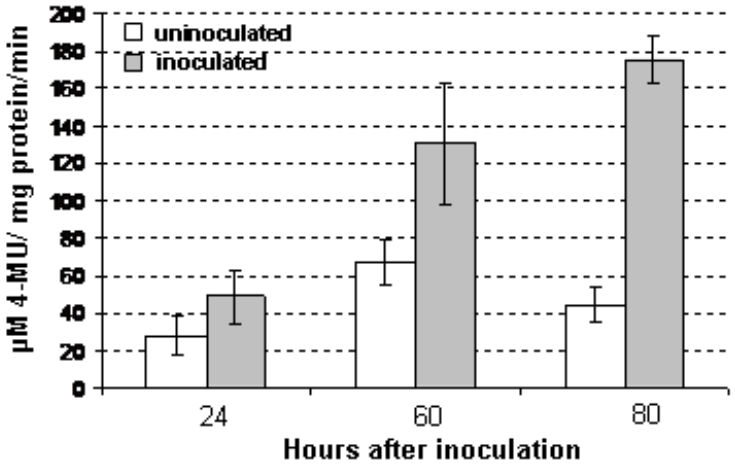

uninoculated
inoculated
µM 4-MU/ mg protein/min
0
20
40
60
80
100
120
140
160
180
200
24
60
80
Hours after inoculation

Figure 7

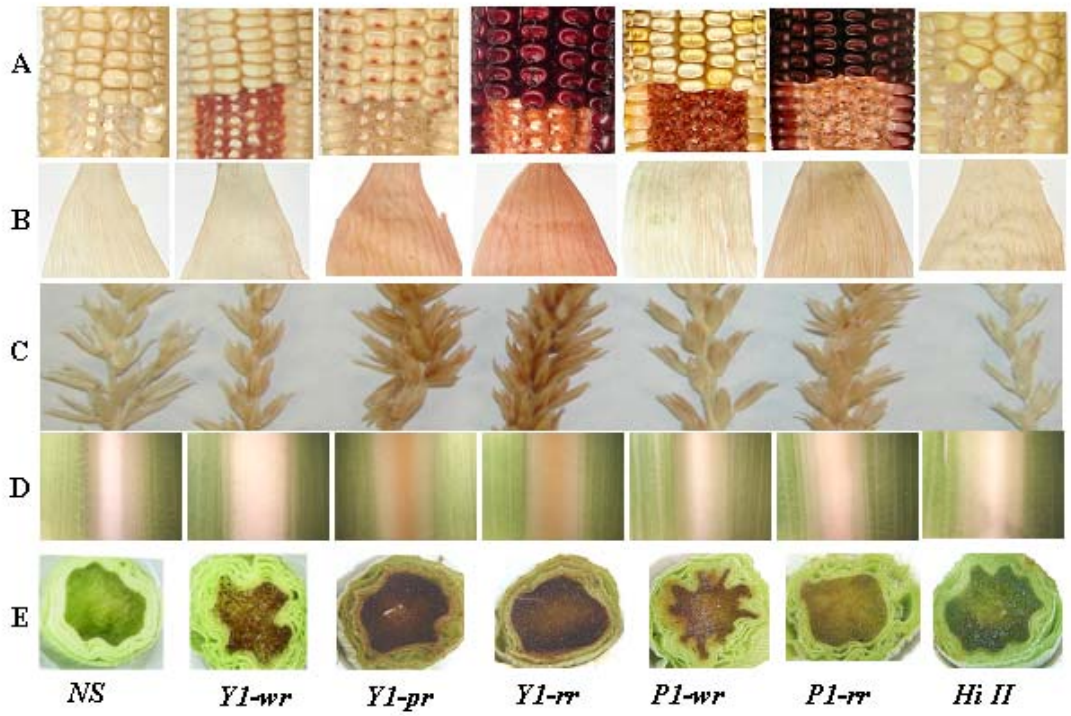
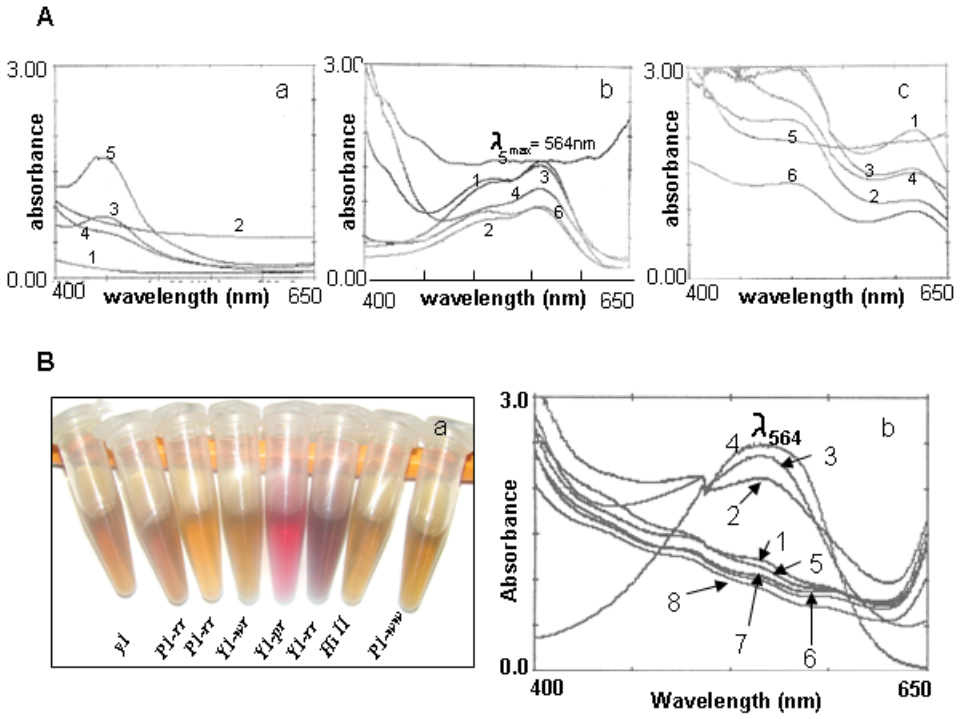


Figure 8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0.00
absorbance
400
wavelength (nm)
650
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b
c
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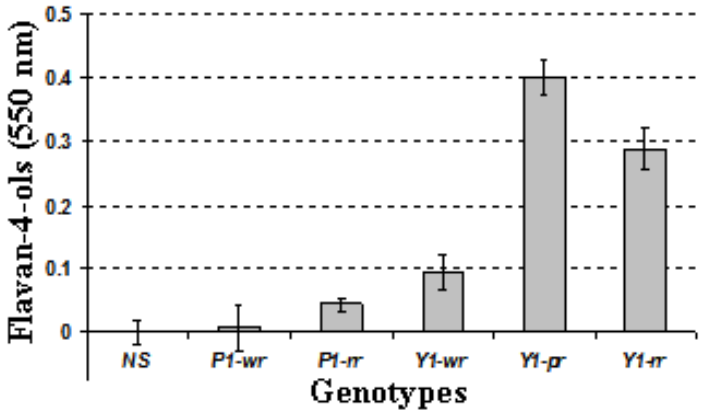
Absorbance
λ564
Wavelength (nm)

Figure 9


Flavan-4-ols (550 nm)
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0.4
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0
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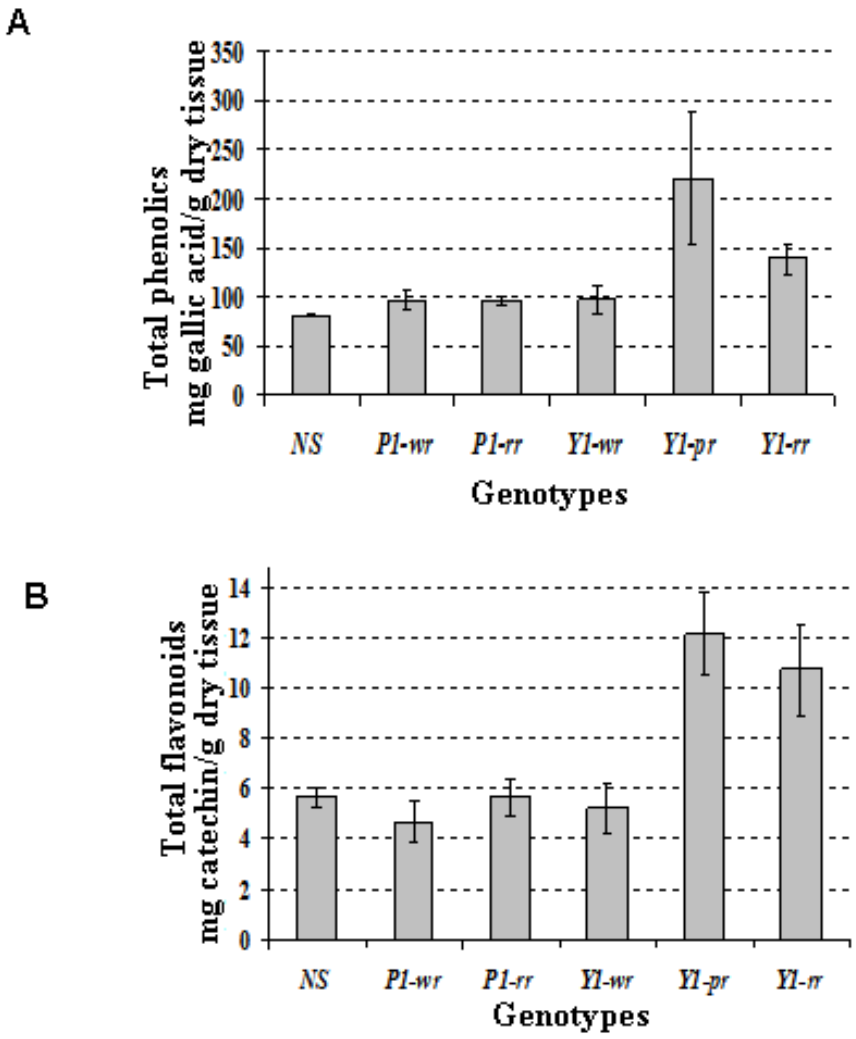
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes

Figure 10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200
150
100
50
0
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P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes
B
Total flavonoids
mg catechin/g dry tissue
14
12
10
8
6
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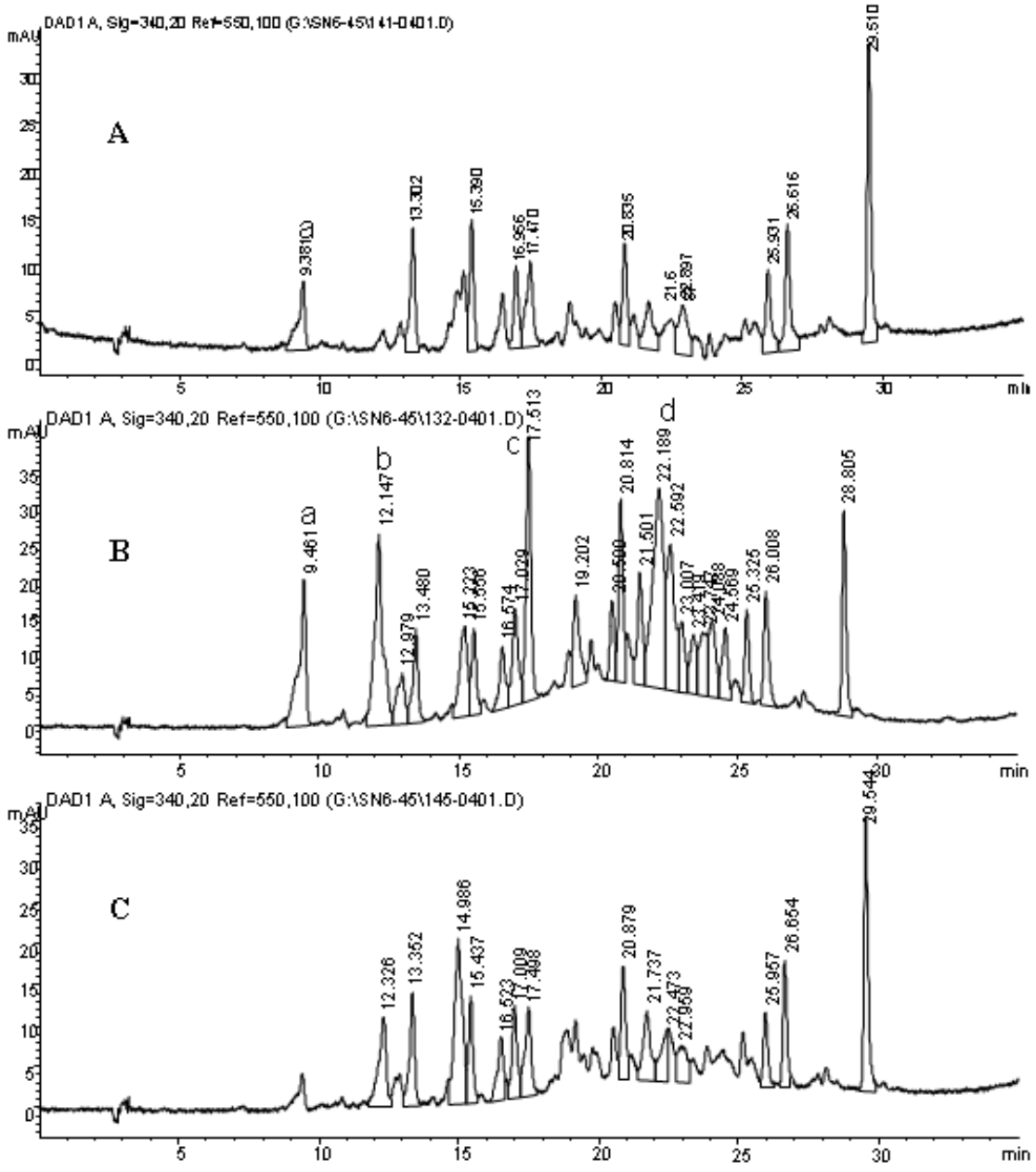
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes

Figure 11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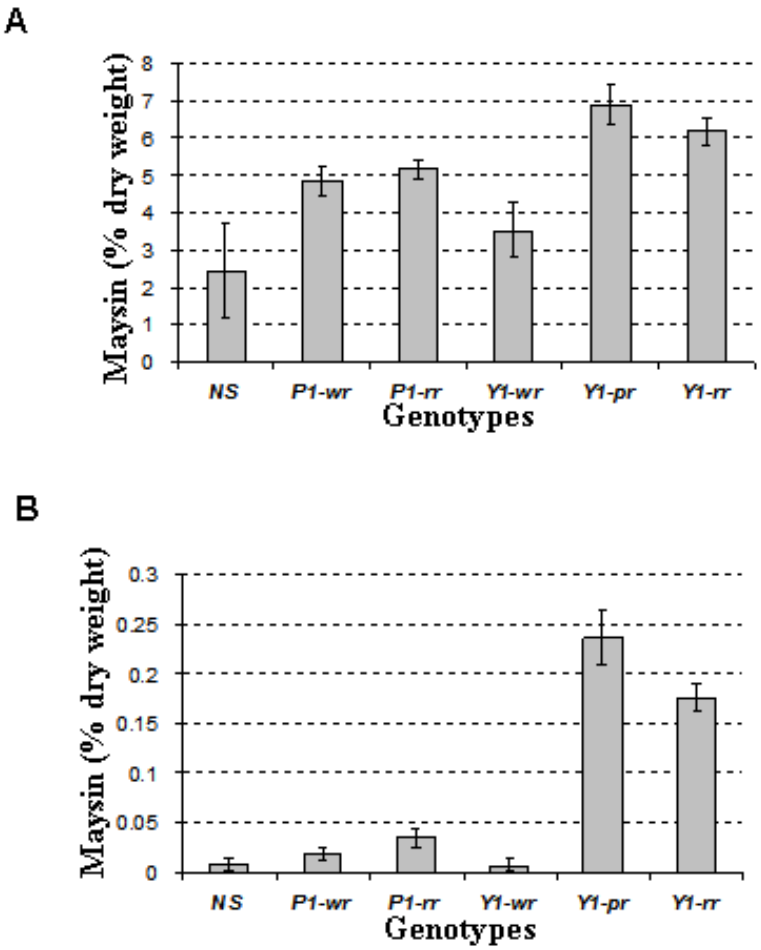
min

Figure 12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Maysin (% dry weight)
8
7
6
5
4
3
2
1
0
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes
B
Maysin (% dry weight)
0.3
0.25
0.2
0.15
0.1
0.05
0
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes

Figure 13

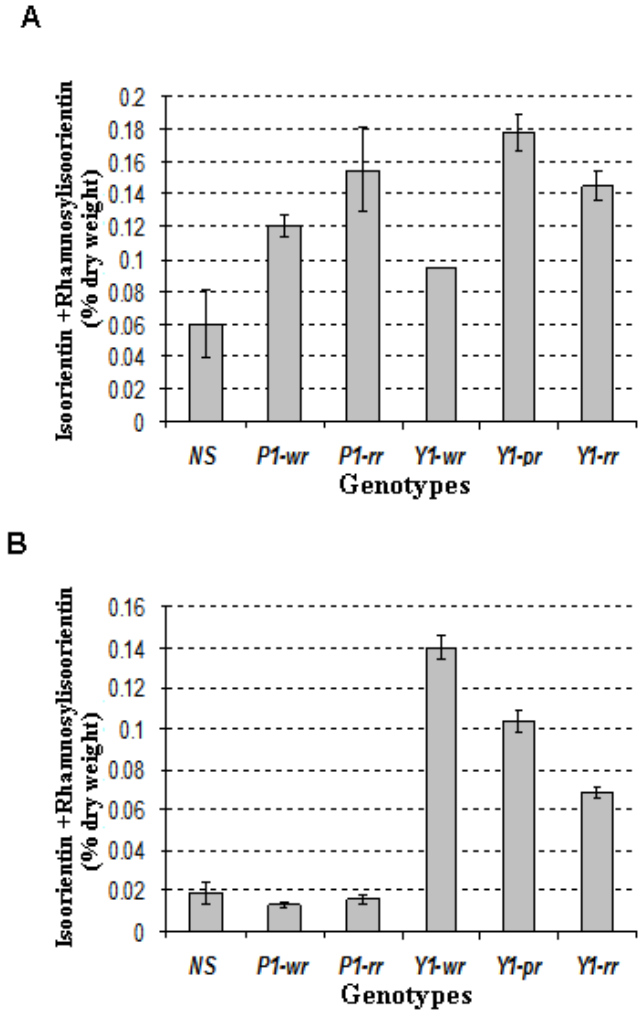

A
Isoorientin +Rhamnosylisoorientin (% dry weight)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes
B
Isoorientin +Rhamnosylisoorientin (% dry weight)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes

Figure 14

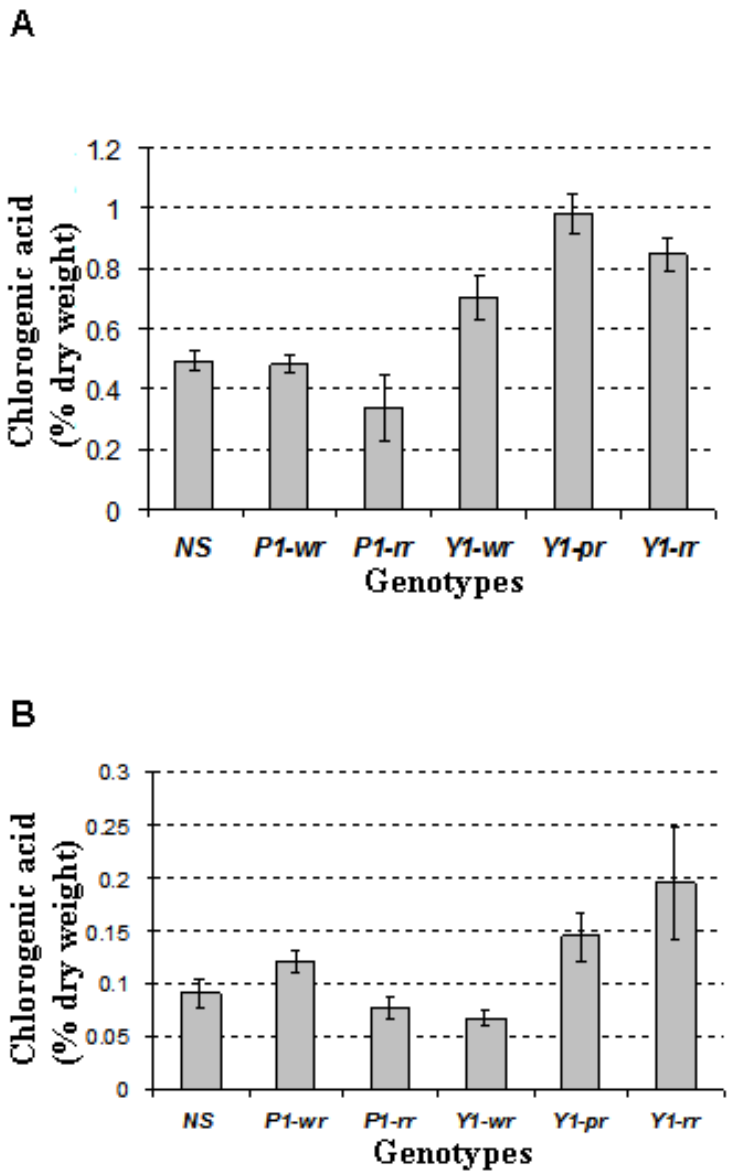

A
Chlorogenic acid (% dry weight)
1.2
1
0.8
0.6
0.4
0.2
0
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes
B
Chlorogenic acid (% dry weight)
0.3
0.25
0.2
0.15
0.1
0.05
0
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes

Figure 15

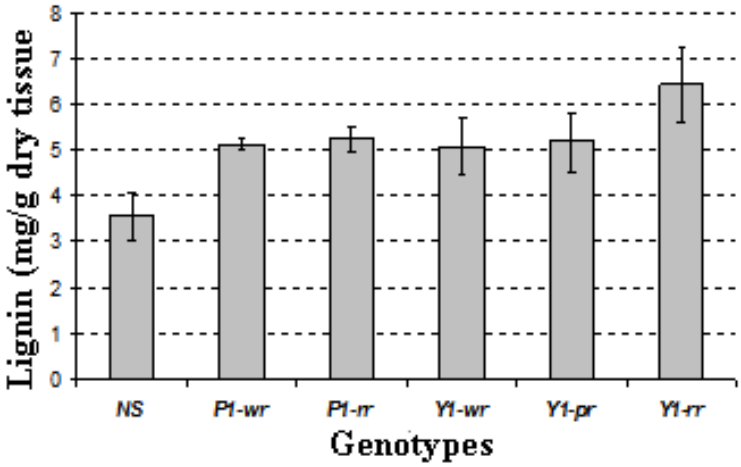
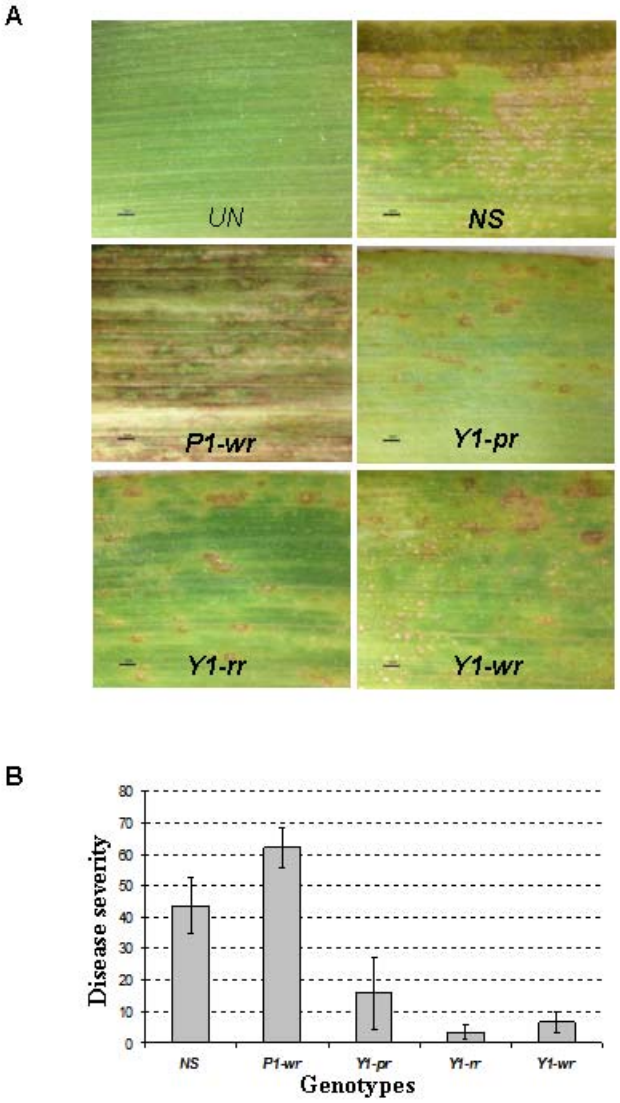

Lignin (mg/g dry tissue
0
1
2
3
4
5
6
7
8
NS
P1-wr
P1-rr
Y1-wr
Y1-pr
Y1-rr
Genotypes

Figure 16


A
UN
NS
P1-wr
Y1-pr
Y1-rr
Y1-wr
B
Disease severity
80
70
60
50
40
30
20
10
0
NS
P1-wr
Y1-pr
Y1-rr
Y1-wr
Genotypes

Chapter 4

Analysis of phytohormone accumulation during 3-deoxyanthocyanidins phytoalexins biosynthesis in sorghum-fungal interaction

Farag Ibraheem and Surinder Chopra

Abstract

Sorghum (*Sorghum bicolor*) responds to infection of pathogenic and non pathogenic fungi by intensive production of 3-deoxyanthocyanidins phytoalexins at the primary infection sites. The potential contribution of these compounds to sorghum defense is well established; however, the signaling cascade upstream their biosynthesis is far from clear. We have previously demonstrated that the *myb* transcription factor *yellow seed1* is required for the biosynthesis of sorghum 3-deoxyanthocyanidins. Sorghum mutant that carries a non-functional version of *y1* loses its ability to synthesize 3-deoxyanthocyanidins indicating that *y1* is an important node in the signaling cascade that leads to the biosynthesis of these compounds. *y1* is induced very early after fungal infection and drives the transcriptional activation of the structural genes such as chalcone synthase (*chs*), chalcone isomerase (*chi*), dihydroflavanol reductase (*dfr*), and flavonoid 3' hydroxylase (*f3'h*) during 3-deoxyanthocyanin biosynthesis. However, the upstream signal that leads to its activation is not known. Phytohormones have been shown to be central components of the signaling network regulating the cross-communication among different plant responses to pathogen attacks. In the current study, we investigated the possible changes in the level of key phyohormones such as salicylic acid (SA), jasmonic acid (JA), indole acetic acid (IAA), and abscsic acid (ABA) during 3-deoxyanthocyanidin phytoalexins biosynthesis. Our analysis indicated that the early induction of sorghum phytoalexins biosynthesis is associated with active changes in the phytohormones analyzed. These changes include early transient accumulation of JA and IAA. In addition, the biosynthesis of sorghum phytoalexins was also associated with limited changes in SA and suppression of ABA. Interestingly, our results revealed that an antagonistic interaction between JA and SA biosynthesis might be active during production of sorghum phytoalexins.

Introduction

Plants have evolved different active defense mechanisms to cope with the continuous challenges of pathogenic and nonpathogenic microbes. These mechanisms include reprogramming cellular metabolism toward strengthening of cell walls, induction of pathogenesis related proteins (PR proteins), and biosynthesis of antimicrobial compounds known as phytoalexins (Kombrink and Somssich, 1997; Rushton and Somssich, 1998). To effectively resist pathogen attack and maintain their fitness, plants are able to induce particular defenses that are specifically active against the pathogen they encounter (Van Loon, 2000). Phytoalexins have been shown in many model systems to be involved in an active defense mechanism against a wide array of pathogens (Hammerschmidt, 1999; Mansfield, 2000). In general, the synthesis of defense related compounds often includes basic cellular processes such as: 1) perception of an extra- or intracellular signal by receptor (s) on the plasma membrane or endomembranes, 2) initiation of a signal transduction cascade that leads to induction of, in most cases, transcription factors, 3) induction of the transcription of structural genes and production of active enzymes, and 4) biosynthesis of the targeted plant defense compounds (Zhao et al., 2005).

Recognition of the pathogen at the primary infection site induces a diverse array of metabolic processes in the host. These include changes in ion exchange via the plasma membrane, production of reactive oxygen species, elicitation of a phosphorylation/dephosphorylation cascade, induction of phospholipases, and biosynthesis of phytohormones such as jasmonic acid (JA), salicylic acid (SA), auxin

(IAA), abscisic acid (ABA) and ethylene (Gachomo et al., 2003). A large body of evidence demonstrated the crucial role of phytohormones in the intricate signal transduction network that leads to the up-regulation of defense related genes (Glazebrook, 2001; Thomma et al., 2001; Gachomo et al., 2003; De Vos et al., 2005; Pieterse and Dicke, 2007; Kunkel and Brooks, 2002).

JA and its related compounds are mainly active in the induction of plant defense against necrotrophic pathogens (Pieterse and Dicke, 2007). Accumulation of JA after infection has been reported in both dicots and monocots. For example, infection of Arabidopsis plants with *Alternaria brassicicola* resulted in significant accumulation of JA in the inoculated leaves and in leaves at a distance from the primary site of infection (Penninckx et al., 1996). In addition, inoculation of tobacco leaves with *Pseudomonas syringae* elicited several fold increase in the level of endogenous JA (Kenton et al., 1999). In rice, inoculation of leaves with *Pyricularia oryzae* (*Magnaporthe grisea*) led to activation of phospholipase A_2, peroxidase and lipoxygenase (enzymes required for JA biosynthesis) and this was correlated with the accumulation of JA precursors (Li et al., 1991). Inoculation of maize leaves with the rough dwarf virus also induced a significant increase in endogenous JA (Vigliocco et al., 2002).

Targets of JA-signaling pathway include *PDF1.2*, a gene encoding an antifungal peptide belonging to plant defensins, *PR-3* and *PR-4.* The expression of these genes was coordinately induced with JA accumulation in Arabidopsis after the infection with *A. brassicicola* and *Botrytis cinerae,* but not with *Peronospora parasitica* (Penninckx et al., 1996; Thomma et al., 1998). Arabidopsis *coi1* mutant plants which are impaired in the

JA-signaling pathway showed a complete suppression of *PDF1.2* and *PR-3*, and significant reduction of *PR-4* expression in response to *A. brassicicola* infection (Penninckx et al., 1996; Thomma et al., 1998).

SA is another key endogenous signaling molecule in the induction of local and systemic acquired resistance (SAR) to several pathogens (Ryals et al., 1996; Durner et al., 1997). In general, SA is active in induction of plant defense mechanisms against biotrophic pathogens, i.e. pathogens that depend on live host cells to survive (Pieterse and Dicke, 2007). Several fold increases in the endogenous levels of SA were reported in Arabidopsis, cucumber, and tobacco in response to *Peronospora parasitica, P. syringae*, and viral infection (Malamy et al., 1990; Metraux et al., 1990; Ryals et al., 1996). In contrast to dicots, bacterial or fungal infections did not elicit significant accumulation of endogenous SA in rice (Silverman et al., 1995; Yang et al., 2004). Also, the exogenous application of SA to rice plants did not induce the expression of PR genes (Yang et al., 2004). These studies suggested that SA might not be directly involved in induced resistance in rice. However, a role for SA in modulating the redox state of rice cells by controlling the level of ROS after infection has been clearly demonstrated (Ganesan and Thomas, 2001; Yang et al., 2004).

Treatment of Arabidopsis leaves with either SA, or inoculation of these leaves with *P. parasitica* or *P. syringe* induced expression of PR genes (Uknes et al., 1992; Uknes et al., 1993). Experiments using mutants with suppressed (*npr1*/*nim1*,/*sai1*) or those with enhanced (*cpr*, *cim*) SAR demonstrated the role of SA as a modulator for the expression of *PR-1*, *PR-2*, and *PR-5* (Thomma et al., 1998; Ham and Bent, 2002). SA

also plays a role in the induction of camalexin biosynthetic genes in Arabidopsis. The biosynthesis of camalexin was impaired in NahG mutant, whereas *npr1*/*nim1* mutants synthesized camalexin in response to PsmES4325. These results suggested that SA is necessary for the induction of camalexin biosynthesis (Zhao and Last, 1996).

ABA has been implicated in modulation of many physiological process in plants under varies types of stress including wounding and pathogen invasions (Anderson et al., 2004; Nambara and Marion-Poll, 2005; Mohr and Cahill, 2007). A large body of evidence has linked high levels of ABA to plant susceptibility to different pathogens. For instance, exogenous application of ABA suppressed the resistance of soybean against *Phytophthora sojae* (McDonald and Cahill, 1999) and *Phytophthora megasperma* (Ward et al., 1989). Furthermore, treatment of Arabidopsis plants with ABA decreased the resistance of these plants against *Pronospora parasitica*. In addition, ABA-deficient Arabidopsis mutant *aba1-1* exhibited enhanced resistance against *P. parasitica* (Mohr and Cahill, 2003). Comparable results have been reported for tomato-*Botrytis cinerae* or tomato-*P.syringae* interactions (Audenaert et al., 2002). Similarly, in monocots significant accumulation of ABA has been reported in susceptible wheat cultivars in response to infection with *Tilletia caries* (Maksimov et al., 2002).

The role of ABA in mediating host response (increasing susceptibility) to the pathogen occurs via suppression of SA- and/or JA-dependant defense mechanisms (Audenaert et al., 2002; Anderson et al., 2004). Exogenous application of ABA has been demonstrated to suppress PAL at the protein and transcription level in soybean and tomato (Ward et al., 1989; Audenaert et al., 2002). In addition, at physiological

concentrations [10^{-4}M], ABA suppressed the expression of β 1, 3 glucanase in tobacco cell cultures (Rezzonico et al., 1998). In addition, ABA has been shown to play a role in monitoring levels of ROS probably via mediating levels of antioxidant intermediates and activities of antioxidant enzymes such as catalase, ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Jiang and Zhang, 2001; 2002).

IAA has been implicated in mediation of plant defense response in addition to its role in regulating other essential process of plant growth and development (Ludwig-Muller et al., 1993; Ludwig-Muller J et al., 1995; Grsic et al., 1999; Maksimov et al., 2002). Like ABA, IAA has been generally implicated in down regulation of plant defense responses. For example, IAA was positively linked to the susceptibility of wheat cultivars to *Tilletia* infection. Its level increased in the susceptible cultivars while it decreased in resistant ones in response to fungal infection (Maksimov et al., 2002). The progress of clubroot disease, a tumorous swelling on the root of cabbage formed in response to infection with *Plasmodeophora brassicae*, was associated with induction of IAA biosynthesis (Ludwig-Muller J et al., 1993).

IAA has been reported to negatively regulate the spontaneous expression of pathoegnesis related proteins such as β 1, 3 glucanase and chintinase (PR2) (Meyer et al., 1984; Leguay and Jouanneau, 1987; Meyer et al., 1987; Leguay et al., 1988; Grosset et al., 1990; Jouanneau et al., 1991). In addition, auxin starvation has been reported to induce chalcone synthesis and glyceollin accumulation (Leguay and Jouanneau, 1987; Leguay et al., 1988). Further, IAA inhibited the wound-induced protease inhibitors in tomato and potato plants (Pena-Cortes et al., 1995).

Signal transduction cascades downstream of the signaling compounds are often mediated by transcription factors. A number of transcription factors that activate defense related genes have been identified and they belong to major families of transcription factors such as WRKY, EREBP, MYB, and bZIP proteins (Eulgem, 2005; Jalali et al., 2006). These regulatory proteins trans-activate their targets genes in the cell's nucleus by binding to specific *cis* recognition sequences within the promoters of these genes. Specific examples include W box (TTGACC) for WRKY proteins; MRE (Myb recognition element, A(A/C)C(A/T)A(A/C)C) for MYB-like proteins; G-boxes (CACGTG) for gene involved in ABA, light, UV, and wounding response; GCC BOX (AGCCGCC) for factors working in ethylene response, and *as-1* elements (CTGACGTAAGGGATGACGCAC) which are responsive to diverse signals such as SA, JA, auxin, and H_2O_2 (Rushton and Somssich, 1998; Garreton et al., 2002). The presence of these recognition sequences in the promoter of many defense-related genes have been well documented using different molecular approaches (Rushton and Somssich, 1998; Eulgem, 2005).

The defense signaling pathways have been reported to cross-communicate (Felton and Korth, 2000; Feys and Parker, 2000; Taylor et al., 2004) . For example, both synergistic and antagonistic interactions between the signaling pathways mediated by SA and JA have been previosly shown (Bostock, 2005). In addition, at physiological levels [10^{-5}M], JA has been demonstrated to interfere with the auxin-dependant responses (Irving et al., 1999). Several regulatory proteins that play a role in controlling such cross talks have been identified. For example WRKY70, a transcription factor in Arabidopsis,

has been suggested to function as a "molecular switch" between JA- and SA-dependant cascades (Li et al., 2004). Both exogenous and endogenous SA induced its expression while an inhibitory effect by JA was reported. Interestingly, the over expression of WRKY70 caused constitutive expression of SA-dependant genes (*PR1, PR2,* and *PR5*) while its suppression activated JA-dependant genes expression (Li et al., 2004). Another example is the NPR1 (Non-expresser of PR1) protein. The *npr1* Arabidopsis plants that are impaired in the SA signaling pathway accumulated high levels of JA and showed an up regulation of JA-dependent genes in response to *Pseudomonas syringae* (Spoel et al., 2003).

Sorghum responds to fungal infection by producing 3-deoxyanthocyanidin phytoalexins. These compounds accumulate early at the primary infection site in the epidermal cell under attack and contribute significantly to sorghum defense against anthracnose fungus (Snyder and Nicholson, 1990; Lo et al., 1999). The biosynthesis of sorghum phytoalexins involves activation of the flavonoid pathway which occurs under the control of a myb transcription factor encoded by *yellow seed1* gene (*y1*, Chapter 2). The upstream signals that precede the activation of the flavonoid pathway during the biosynthesis of these compounds are still unknown. To the best of our knowledge, biochemical analysis of the concomitant changes in any of the signaling compounds during sorghum phytoalexin biosynthesis has not been previously reported**.** Thus in the current chapter we report our investigations of changes in phytohormones during sorghum phytoalexin biosynthesis. Our results indicated that the onset of phytoalexin biosynthesis is associated with early accumulation of JA and IAA. On the other hand,

there was either no change or a suppression of SA and ABA levels. In addition, our data demonstrated that an active negative correlation between endogenous levels of JA and SA is present during sorghum phytoalexin biosynthesis.

Materials and methods

Plant and fungal materials

A sorghum line carrying *Y1-rr3* allele was used in this investigation. This line was derived from a candystripe progenitor line [CS8110419] that carries a mutable *Y1-cs30* (candystripe) allele (Chopra et al., 1999). The fungus, *Cochliobolus heterostrophus* **(**anamorph *Bipolaris maydis* [Nisikado and Miyake] Shoemaker**)** was grown on potato dextrose agar (PDA) under constant fluorescent light at room temperature for 10 days. The fungal conidia were scraped from fungal colonies and collected in a tween/water mixture as described previously (Lo and Nicholson, 1998; Aguero et al., 2002). Conidial suspensions were filtrated through cheese cloth and diluted to get a concentration of 10^6 spores ml^{-1}.

Plant growth & fungal inoculation conditions

Seeds were surface sterilized for 1 h with 10% commercial bleach, rinsed with sterile water and then imbibed for 12 h. To get uniformly etiolated seedlings, seeds were planted in rolls of paper towels and geminated in the dark for 5 days at 26°C in a growth chamber. Phytoalexin biosynthesis was initiated by inoculation of seedlings with *C. heterostrophus*. This fungus was chosen because it induces a rapid and intensive

accumulation of phytoalexins during its attempted penetration of sorghum tissues (Lo and Nicholson, 1998; Aguero et al., 2002). For inoculation, a fungal spore suspension was applied onto etiolated seedlings followed by incubation in the dark in a growth chamber adjusted at 26°C and 100% relative humidity. For controls, etiolated seedlings were sprayed with tween/water mixture only. Triplicate bulk samples were collected from inoculated and uninoculated seedlings at 0, 6, 12, 24, 36, and 48 hours post-inoculation (hpi). At the time of tissue collection, seedlings were excised 5 mm above the point of attachment to the seed and the upper part of seedling was collected. The collected tissues were immediately dipped in liquid nitrogen, grounded into powder and stored at -80°C for phytoalexin and phytohormones analysis.

Spectrophotometery

Spectrometric analysis was conducted to detect the presence of sorghum phytoalexins in the inoculated *Y1-rr3* seedlings at different time points after inoculation. The powdered tissues harvested at the time points indicated above were incubated in 0.083% HCl in HPLC grade methanol at 4°C for 24 h. Consequently, the methanolic extracts were centrifuged at 10,000 rpm for 10 minutes and the clear supernatants were collected in fresh tubes and used for qutification of total 3-deoxyanthocyanidins. The content of total 3-deoxyanthocyanidin phytoalexins in the extracts was measured spectrophotometrically as described previously (Nicholson et al., 1987; Yamaoka et al., 1990). Briefly, absorbance of the methanolic extracts at 480 nm was recorded using 1 cm wide quartz curvet and a UV mini-1240 spectrophotometer. The concentration of

phytoalexins was expressed as μM of phytoalexins based on the Lambert-Beer Law using the molar extinction coefficient of luteolinidin (13800 $M^{-1}cm^{-1}$) (Aguero et al., 2002).

Analysis of phytohormones

Phytohormones were analyzed in the same tissues used for phytoalexin analysis to link any temporal changes in the level of these compounds to the content of 3-deoxyanthocyanidin phytoalexins. The levels of salicylic acid (SA), jasmonic acid (JA), Abscisic acid (ABA), and indole acetic acid (IAA) were measured simultaneously using chemical ionization gas chromatography mass spectrometry as described recently (Engelberth et al., 2003; Schmelz et al., 2003). This protocol depends on converting the phytohormones to their corresponding methyl esters which are then measured as volatiles using GC-MS.

For phytohormone extraction, 100 mg of powdered sorghum tissues was mixed with 300 μl 1-propanol/H_2O/HCl (2:1:0.002, v/v) in a 2-ml glass vial containing 1 g of Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ), dihydro JA, and [$^2H_6$] SA (100 ng). The tube contents were shaken in a FastPrep FP 120 tissue homogenizer (Qbiogene) for 30 s then 1ml dichloromethane was added and mixture was reshaken for another 10 s. After centrifugation for 30 s at 10,000 Xg, the bottom layer (dichloromethane/1-propanol) was carefully transferred to 4-ml screw-cap glass vial. This organic phase was then evaporated to total dryness using constant airstream and 100 μl of diethyl ether/ methanol (9:1, v/v) was added. Subsequently, 2 μl of a 2.0 M trimethlsilydiazomethane in hexane was used to convert carboxylic acids in the extract to

their corresponding methyl esters. The vials were then closed, vortexed, and incubated at room temperature for 30 min. 2 µl of a 2.0 M acetic acid solution was added and the tubes were incubated for another 30 min to destroy excess trimethlsilydiazomethane. Vials were then heated to complete dryness on a hot plate and the volatile compounds were trapped in a Super Q filter. The trapped compounds were then eluted from the filter with 150 µl dichloromethane and quantified using chemical ionization GC/MS.

Dissection of *y1* promoter

The analysis of the *y1* promoter for the location and distribution of *cis* regulatory sequence elements were performed using the Plant Care database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Statistical analysis

Statistical analysis was performed via analysis of variance (ANOVA) using the mixed procedure (PROC MIXED) (SAS Institute, 1999). The difference between groups was considered statistically significant when a P value was < 0.05.

Results

The induction of plant defense mechanisms is controlled by a network of multiple components that may work in parallel or interconnected pathways. The results reported here are not intended to give a complete quantitative description of sorghum-*C.*

heterostrophus interaction but rather to describe the changes in a number of phytohormones at different time points after fungal inoculation.

In chapter 2, we established the essential role of the *yellow seed1* gene (*y1*) in controlling the biosynthesis of 3-deoxyanthocyanidin phytoalexins in sorghum. We also demonstrated the induced expression of *y1* at the RNA level. Here we first characterized the promoter of *y1* for the presence of specific *cis*-regulatory elements by using the Plant Care database. The *y1* promoter contains multiple *cis* acting elements identical to those identified in many plant defense genes. Locations of these elements are illustrated in Table 1 and a set of known sequences of defense related genes are shown in Figure 1. An elicitor responsive element (EIRE), TTCGACC, is present in the coding strand at position -500 bp. In addition, 5 W boxes with TGAC core sequences are located in both coding and non-coding strands. These boxes are arranged in a very specific manner within the *y1* promoter (Fig. 1). Further, several regulatory elements that have been shown to mediate the transcriptional regulation of genes involved in methyl jasmonate- (CGTCA, TGACG), wounding- (TCATTACGAA), and ABA-(TACGTG, TGCCGT, CCCACG, CCGCCCCCGCT) response are also present in the 5' regulatory regions of *y1*. Interestingly, many other *cis* sequences that have been shown to be involved in abiotic stress are also present in the *y1* promoter (table 1).

Based on the promoter sequence characterization we conducted simultaneous time course analyses of 3-deoxyanthocyanidin phytoalexins and a number of signaling compounds such as JA, SA, ABA, and IAA in sorghum seedlings in response to infection with *Cochliobolus heterostrophus*. These experiments were designed such that

two aliquots of a treatment were used for phytoalexin and phytohormone analysis to ensure that the observed alterations in the level of signaling compounds would reflect the actual temporal changes in sorghum physiology during phytoalexin biosynthesis.

Fungal infection induces early accumulation of 3-deoxyanthocyanidins

Induction of 3-dexyanthocyanidins is shown in Figure 2. Six hours post inoculation (hpi), sorghum seedlings accumulated trace amounts of 3-deoxyanthocyanidin phytoalexins and by 12 hpi, low but detectable amounts were present. Thereafter, the level of sorghum phytoalexins showed a rapid increase of 9 to 15 fold by 36 hpi. The content of 3-deoxyanthocyanidins then decreased at 48 hpi to a level similar to that at 24 hpi. The uninoculated seedlings did not show any detectable levels of 3-deoxyanthocyanidins at any time point during these experiments which were replicated three times. These results indicated that there was a time lag of about 12 h after fungal inoculation before detectable levels of 3-deoxyanthocyanidin phytoalexins accumulated in sorghum seedlings.

Fungal infection induces transient increase in JA level

Time course analysis revealed that the biosynthesis of sorghum phytoalexins is associated with dynamic changes in the level of phytohormones shown to mediate plant defense against pathogens. The basal level of JA in sorghum seedlings was about 20 ng g^{-1} fresh weight (Fig. 3). Throughout the experiment, the JA level in the uninoculated seedlings fluctuated within a range of about 20 to 32 ng g^{-1} compared to 18 to 50 ng g^{-1} in

the inoculated one. In the infected seedlings, the JA levels showed little but significant increase at 6 hpi ($P = 0.0025$), reached a maximum at 12 hpi (~ 48 ng g^{-1}, $P < 0.0001$) and remained high during the subsequent time points. However, unexpected decrease in JA level (~ 18 ng g^{-1}) was observed at 24 hpi. In summary, in response to fungal infection, sorghum seedlings had a general tendency to maintain a significantly higher level of JA.

Fungal infection induces limited changes in SA level

Sorghum seedlings have a basal SA level of 34 ng g^{-1} fresh weight (Fig. 4). This was the highest level detected for SA in the inoculated and uninoculated sorghum seedlings throughout the course of the experiment. In the control seedlings, the SA level dropped significantly from the basal level with more than 50% decrease at 6 h from the beginning of the experiment. Thereafter, the SA level fluctuated within a range of 12 to 20 ng g^{-1} during the experiment. The inoculated seedlings had SA levels similar to that of control seedlings at all time points tested expect at 24 and 36 hpi. The most obvious difference between inoculated and uninoculated seedlings was observed at 24 hpi where the inoculated seedlings showed a transient increase in SA (~23 ng g^{-1}, P <0.0001) compared to that of the control seedlings (~9 ng g^{-1}.). At 36 hpi, the inoculated seedlings maintained significant higher level of SA than the control seedlings; however, the extent of such difference was much lower than at 24 hpi ($P = .0338$). It is interesting to note that the SA levels after inoculation never exceeded its basal level in sorghum seedlings.

Fungal infection induces transient increase in IAA

Sorghum seedlings had a basal IAA content of about 13 ng g^{-1} fresh weight (Fig. 5). IAA levels then fluctuated in the control seedlings throughout the experiment. Fungal-inoculated seedlings exhibited significant accumulation in the endogenous IAA level at 6 ($P = 0.0001$) compared to uninoculated seedlings. However, insignificant differences in IAA levels were observed between these seedlings at 12, 24, 36, and 48 hpi compared to the control seedlings.

Fungal infection elicites reduction in ABA

Prior to inoculation, sorghum seedlings accumulated 25 ng ABA g^{-1} fresh weight (Fig. 6). Both the inoculated and uninoculated seedlings showed fluctuation in the level of ABA throughout the experimental period. The highest levels of ABA were observed in control seedlings at 0, 6 and 48 hpi. Fungal inoculation resulted in a significant reduction in ABA levels at 6 ($P < 0.0001$) and 48 hpi ($P = 0.089$). No significant changes were observed in the ABA levels between inoculated and uninoculated seedlings at 12 ($P = 0.1865$), 24 ($P = 0.0605$) and 36 hpi ($P = 0.3432$).

Discussion

When plants are under attack, defense systems are regulated through a complex network of different signaling pathways. Plants have been equipped with built-in strategies to organize the cross talk among these defense mechanisms such that they can turn on the suitable defense mechanism against the pathogen they encounter. Sorghum

responds to diverse fungal infections by a rapid and intensive production of 3-deoxyanthocyanidin phytoalexins (Snyder and Nicholson, 1990; Lo and Nicholson, 1998; Aguero et al., 2002). The rapid biosynthesis of sorghum phytoalexins in response to fungal attack demonstrates an active defense system involving coordination of signaling pathways of multiple components. Although much work has been focused on the characterization of these compounds and establishing their roles in sorghum defense against pathogens, very little is known about the upstream signals that precede the synthesis of these compounds. In general, phytohromones are crucial components of the intricate signal transduction network that regulates the induction of general plant defense mechanisms (Glazebrook, 2001; Thomma et al., 2001; Kunkel and Brooks, 2002). In the current study, we quantified the levels of JA, SA, IAA, and ABA as well as the content of 3-deoxyanthocyanidin phytoalexins during the sorghum-*C. heterostrophus* interaction.

C. heterostrophus is a filamentous, heterothallic fungus that belongs to the Ascomycota. It infects plant tissues through either stomata or penetrates directly via epidermis. The process of penetration takes about 6 hours (Yoder, 1997; Dias et al., 2003). This fungus has been shown to induce intensive accumulation of 3-deoxyanthocyanidins during its attempted penetration of sorghum tissues. Our current investigation indicated that sorghum seedlings started to accumulate 3-deoxyanthocyanidins phytoalexins during early stages of sorghum-fungus interaction. Active changes in the levels of the signaling molecules recorded in the current investigation indicate that sorghum seedlings rapidly detect the presence of the fungus once it begins to penetrate the tissue. This suggests that the initial events of fungal

penetration initiate a signal transduction cascade which eventually leads to the activation of the flavonoid pathway in sorghum to produce 3-deoxyanthocyanidin phytoalexins. This signaling cascade might have at least three main nodes. *y1* is an important node of this cascade and functions downstream to drive the flavonoid pathway toward the biosynthesis of 3-deoxyanthocyanidins (Chapter 2). The first node works very early in the recognition of the fungal attack. Another one or more nodes work in transmitting this signal to activate *y1*.

Our phytohormone analysis indicated that the biosynthesis of sorghum flavonoid phytoalexins was preceded by induction of JA biosynthesis and accumulation. It is well established that the fungus-induced JA biosynthesis occurs through *de novo* synthesis rather than liberation from JA conjugates (Creelman and Mullet, 1997). Such biosynthesis starts with hydrolysis of membrane lipids, in a reaction catalyzed by certain lipases, to linoleic acid which is then metabolized via multi-step pathway to produce JA (Schaller, 2001). The biosynthesis of JA should be coupled with activation of genes/enzymes working in the JA biosynthetic pathway. Such activation might also be attained by suppression of inhibitors interfering with JA biosynthetic genes. In tomato and potato, JA biosynthesis was up-regulated on perception of systamin, a polypeptide that is liberated on wounding of the tissues (Van Loon, 2000). In the current study, the early induction of JA after inoculation with *C .heterostrophus* indicates that the fungus or fungal elicitor has been recognized by a particular sorghum receptor(s) and such recognition generated a signal that leads to the activation of a membrane bound lipase to initiate JA biosynthesis. A large body of evidence has implicated the leucine rich repeat

(LRR) receptor kinases in early recognition fungal elicitors. In sorghum, it has been reported that inoculation of sorghum seedlings with *C. graminicola*, another fungus that stimulates the biosynthesis of the same set of sorghum phytoalexins, induces accumulation of a SLRR-encoding cDNA (Hipskind et al., 1996). This SLRR has high homology with the extra-cellular binding domain of the Arabidopsis receptor-protein kinases RLK5 and TMK1. It is possible that during sorghum-*Cochliobolus* interaction, the same or another LRR recognizes the fungus or fungal elicitor and generates the first signal in the cascade.

The role of jasmonate as a signal modulator in biosynthesis of defense related compounds in general and in flavonoid phytoalexins in particular has been demonstrated in monocots and dicots. For example, the fungus-, and elicitor-induced flavonoid phytoalexin sakuranetin, in rice leaves was associated with the transient increase in the endogenous levels of JA. In addition, the exogenous application of JA to these leaves elicited sakuranetin accumulation (Rakwal et al., 1996; Tamogami et al., 1997). These results indicated that JA represents an important node in signal transduction cascade during the biosynthesis of rice flavonoid phytoalexins. In dicots, similar role of JA as an inducer of flavonoid phytoalexins has also been demonstrated. For example, exogenous application of methyljasmonate or 12-oxo-phytodienoic acid, a JA precursor, to parsley (*Petroselinum crispum)* cell culture induced the accumulation of furanocoumarin phytoalexins and the flavonoid phytoalexin apiin (Dittrich et al., 1992; Ellard-Ivey and Douglas, 1996). The accumulation of apiin was correlated with activation of PAL, 4CL (4-coumarate: CoA), and CHS. In grape (*Vitis vinifera*) cell suspension cultures, leaves,

and berries, the exogenous application of methyl jasmonate induced the accumulation of a stilbene phytoalexin (Larronde et al., 1998; Larronde et al.). In addition, treatment of soybean hypocotyls and petunia corollas with JA induced accumulation of *chs* and *dfr* transcripts as early as 3 to 5 h after treatment (Creelman et al., 1992; Tamari et al., 1995). These results support the hypothesis that JA might play a role in regulating flavonoid biosynthesis at the transcriptional level.

In sorghum, the accumulation of the flavonoid structural genes, *chs*, *chi*, *dfr* and *f3'h* transcripts precedes the biosynthesis of 3-deoxyanthocyanidins phytoalexin and such accumulation requires a functional *y1* (Chapter 2). Our current investigation indicated that the biosynthesis of sorghum phytoalexin is preceded with transient accumulation of endogenous JA. In addition, the sorghum line that has a null *y1* showed accumulation of JA at 24 hpi, but this line neither showed accumulation of flavonoid structural genes transcript nor produced appreciable levels of 3-deoxyanthocyaninidin in response to fungal attack. Further, this line showed intensive colonization by the fungus at 32 hpi (Chapter 2, Fig. 7A and B). These observations might suggest that JA and *y1* are components of the same defense mechanism that involves biosynthesis of 3-deoxyanthocyanidin phytoalexins and dense against pathogens. Thus it is possible that the fungus-induced JA stimulates the transcription of the flavonoid structural genes by activating *y1*. In fact, mining databases revealed that the 5' regulatory regions upstream of the *y1* transcription start site contains two *cis* recognition sequences similar to those identified in the JA-responsive genes (Rouster et al., 1997). It is speculative but possible that the fungus-induced JA induces *y1* expression via specific JA-induced protein(s)

(JIP). Another possibility could be that the fungus-induced JA activates *y1* through a particular JA-induced WRKY protein(s). These proteins recognize W boxes in the promoter of their targets and turn on their transcription. Our analysis of the *y1* promoter revealed the presence of 5 W boxes; therefore, it is possible that JA-induced WRKY proteins recognize one or more W boxes in the *y1* promoter and turn on its expression. Interestingly, exogenous application of JA to solution culture-grown sorghum seedlings induced expression of a WRKY like gene (CF772823) and another WRKY–like homolog (BM328251) (Salzman et al., 2005). Once *y1* is activated, it trans-activates the expression of flavonoid structural genes and drives the sorghum flavonoid pathway towards 3-deoxyanthocyanidin biosynthesis (chapter 2). A very similar scenario has been described during induction of sesquiterpene phytoalexins. In cotton, *CAD1* encodes (+) -δ-cadinene synthase, a sesquiterpene cyclase which is required for the biosynthesis of sesquiterpene phytoalexins. Its promoter contains multiple W boxes. It has been demonstrated that both the fungal elicitor and methyljasmonate, but not SA or H_2O_2, up-regulate the expression of *GaWRKY1* and *CAD1*. In addition, the direct interaction between *GaWRKY1* and W boxes in the *CAD1* promoter has been demonstrated (Xu et al., 2004).

The biosynthesis of sorghum phytoalexins was also correlated with early transient accumulation of IAA. As mentioned above, IAA is generally linked to the plant susceptibility to pathogens. It is reported that IAA down regulates the spontaneous expression of a number of PR proteins (Meyer et al., 1984; Leguay and Jouanneau, 1987; Meyer et al., 1987; Leguay et al., 1988; Grosset et al., 1990; Jouanneau et al., 1991). In addition, the accumulated IAA in susceptible cultivars has been suggested to inhibit

peroxidase activities and enhance cell wall loosening and thus facilitates the fungal penetration (Maksimov et al., 2002). In the current study, it is unclear whether the biosynthetic source of the reported increase in IAA is the plant or the fungus or both. Thus it is possible that the fungus either synthesizes IAA or interferes with the IAA biosynthetic pathway in sorghum to facilitate its colonization of the host (Gunasekaran and Weber, 1972; Oku and Shiraishi, 1994). However, the plant might employ such increase of IAA in inhibiting some defense mechanisms, at least during early stages of infection, for the advantage of more potent defense mechanisms such as phytoalexins. Sorghum flavonoid phytoalexins are a mixture of compounds, which make it difficult for a pathogen to build up resistance against them. In addition, they have high antioxidant activity, high stability against pH changes, and high and broad antimicrobial activity (Awika et al., 2004). The frequent, rapid, and intensive production of these compounds suggests that sorghum selects to invest part of the available resources for their production during early stages of plant-pathogen interaction. Therefore, it is possible that sorghum seedlings employ IAA to temporally suppress other defense mechanisms such as the production of PR proteins for the advantage of the production of 3-deoxyanthocyanidins. Another interesting point is that IAA has been reported to increase pathogen infestation by loosing plant cell wall. Sorghum plants might nullify such phenomenon through JA, which has been reported not to react directly with IAA but rather hinders the physiological processes involved in the induction of IAA responses (Saniewski et al., 2002). For example, JA inhibits the IAA-induced elongation of maize coleoptiles by increasing the cytosolic pH (Irving et al., 1999). In addition, JA blocks the IAA-induced

elongation in oat coleoptiles by inhibiting the incorporation of glucose into the cell wall (Ueda et al., 1994; Ueda et al., 1995; Miyamoto et al., 1997) thereby interfering with its mechanical properties. Consistent with the above-mentioned hypothesis, our analysis revealed that fungal inoculation did not induce significant changes in endogenous levels of SA, a potential inducer of a number of PR proteins. These results are in agreement with other reports which demonstrated that fungal and bacterial infection of rice did not induce significant changes in endogenous SA levels (Silverman et al., 1995). The observed limited changes of SA levels in sorghum in response to fungal attack might support the hypothesis that SA does not play an essential role during the sorghum-*Cochliobolus* interaction and 3-deoxyanthocyanidin biosynthesis. Interestingly, exogenous and endogenous SA has been reported to inhibit JA biosynthesis by inhibiting both the expression and the activity of allene oxide synthase (AOS), an important enzyme working early in JA biosynthesis (Raskin, 1992; Doares et al., 1995; Pan et al., 1998; Iverson et al., 2001). Thus it is possible that, during fungal infection, sorghum suppresses SA biosynthesis for two main reasons: first, to hold the induction of PR proteins during the early stage of sorghum-fungus interaction and second, to minimize its inhibitory effect on JA biosynthetic genes/enzymes. As a result, sorghum directs more of its recourses for the biosynthesis of more potential defense mechanism such as phytoalexins. It is not known if fungus-induced JA plays a role in inhibiting SA biosynthesis in sorghum. However, in other model systems such as Arabidopsis, the *coi1* mutant has increased level of SA than the wild type (Kloek et al., 2001).

The biosynthesis of sorghum flavonoid phytoalexins was also correlated with general suppression of ABA biosynthesis. These results are consistent with the reported negative interaction of ABA with flavonoid and phenylpropanoid biosynthesis. As mentioned above, ABA suppresses these metabolic pathways by inhibition of PAL both at the protein and mRNA levels (Ward et al., 1989; Audenaert et al., 2002). In addition, ABA inhibits the deposition of ferulic and diferulic acids on the cell wall of dark grown wheat seedlings (Wakabayashi et al., 1977). ABA also suppressed the biosynthesis of glyceollin phytoalexins (Ward et al., 1989). Whether there is a direct interaction between ABA and JA in sorghum is not yet clear. Our data suggest that JA biosynthesis is associated with suppression of ABA biosynthesis. In Arabidopsis, *AtMYC2* encodes a protein with a BHLH domain and is a positive regulator of ABA signaling pathway. Consistent with our findings, *Atmyc2* Arabidopsis mutants showed higher levels of expression of JA-dependant defense genes and exhibited enhanced resistance to the necrotrophic fungus *Fusarium oxysporum.* In addition, ABA deficient mutants or ABA treated wildtype Arabidopsis plants attained high levels of expression of JA-dependent genes such as *PDF1-2* and *PR4*. These results support the idea that ABA has a negative effect on the JA signaling pathway (Anderson et al., 2004).

In conclusion, the observations reported here suggest that in sorghum, like rice, SA might not play an essential role in signaling during 3-deoxyanthocyanidin biosynthesis and the early stages of response to *C. heterostrophus*. In contrast, JA might be the second main node and act as a signal modulator that connects the recognition of the fungus or fungal elicitor to the activation of *y1* and consequently the induction of

sorghum phytoalexin biosynthesis. Consistent with these findings, it has been suggested that fungal hyphae and insect stylets have similar effects during fungal infection and insect feedings (Fidantsef et al., 1999). However, sorghum mutants in the biosynthesis of JA and SA, or pharmacological studies are required to draw more definitive conclusion from the current data.

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Figure legends

Figure 1. Structural features of *y1* promoter.

The upper panel is a diagrammatic representation of the structure of the *y1* gene. A bent arrow shows the transcription start site. Black boxes represent exons that are connected by lines representing introns. Restriction enzymes are: K, *Kpn*I; SC, *Sca*I; H, *Hin*dIII. B, *Bam*HI, and E, *EcoR*I. TSS is the transcription start site. The lower panel is an illustration of the *cis* recognition elements discussed in the text and are indicated as follows: The rectangular black box represents the TATA box, the rectangular green boxes depicts W boxes, the rectangular blue boxes represent ABA responsive elements, the yellow diamonds represent methyl jasmonate responsive elements, and the red triangle symbolizes the elicitor responsive element. The arrows above and below the panel indicate the orientation (direct or reverse) of the *cis* element.

Figure 2. Spectrophotometric analysis of total 3-deoxyanthocyanidin phytoalexins in inoculated sorghum seedlings carrying *Y1-rr*-3 allele. Phytoalexin concentration is expressed as μM. Values are the means of three biological replicates ± SE at each time point.

Figure 3. Accumulation of total jasmonic acid in sorghum in response to *C. heterostrophus.* Shown are mean values of three replicates ± standard error.

Figure 4. Accumulation of salicylic acid in sorghum in response to *C. heterostrophus.* Shown are mean values of three replicates ± standard error.

Figure 5. Accumulation of indoleacetic acid (IAA) in sorghum in response to *C. heterostrophus.* Shown are mean values of three replicates ± standard error.

Figure 6. Accumulation of abscisic acid (ABA) in sorghum in response to *C. heterostrophus.* Shown are mean values of three replicates ± standard error.

Figure 7. Accumulation of total total JA, 3-deoxyanthocyanidin pigmenst and fungal development in sorghum line that carries null *y1* allele (*y1-ww*).

A. Accumulation of total jasmonic acid in sorghum in response to *C. heterostrophus.* Shown are mean values of three replicates ± standard error.

B. Development of *C. heterostrophus* within *y1-ww* seedlings. The growth of *C. heterostrophus* within inoculated seedlings was investigated, at 32hpi, according to the method described by Vogel et al. (2000). The fungal hyphae within the infected *y1-ww* tissues (B-a) were visualized by staining with 250 ug/ml trypan blue in a mixture of lactic acid, glycerol, and water (1:1:1) for 15 min. The stained tissues were rinsed with the same mixture without trypan blue then tissues were examined under a light microscope connected to a camera. Sorghum line with functional *y1* (*Y1- rr*) was included for comparison (B-b). The red pigments in *Y1-rr* are the 3-deoxyanthocyanidin phytoalexins. These pigments are absent in *y1-ww*.

Figure 1

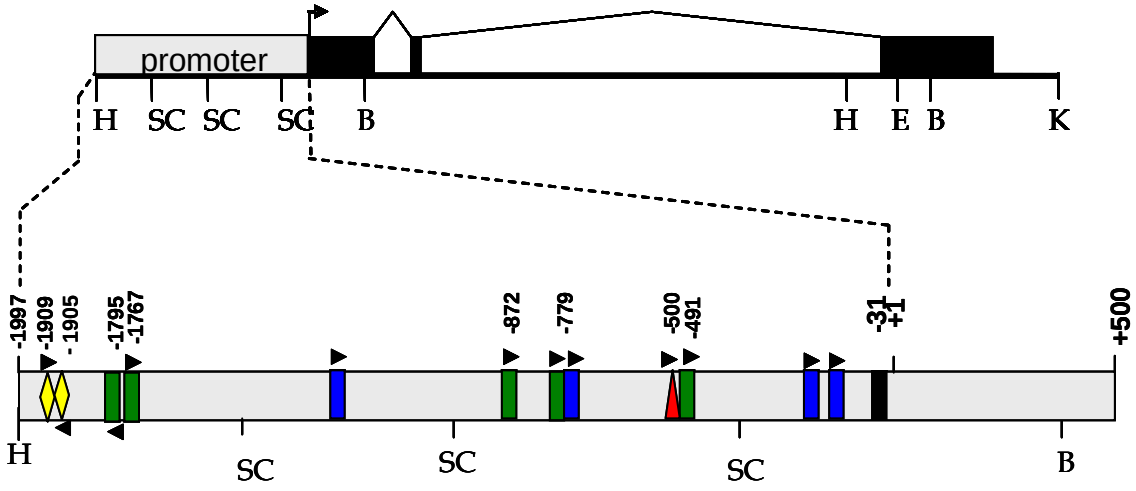

promoter
H
SC
SC
SC
B
H
E
B
K
-1997
-1909
-1905
-1795
-1767
-872
-779
-500
-491
-31
+1
+500
H
SC
SC
SC
B

Figure 2

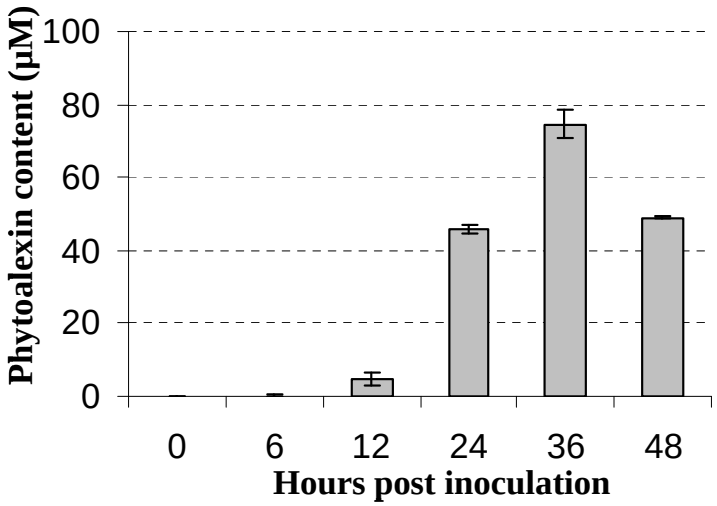

Phytoalexin content (µM)
100
80
60
40
20
0
0
6
12
24
36
48
Hours post inoculation

Figure 3

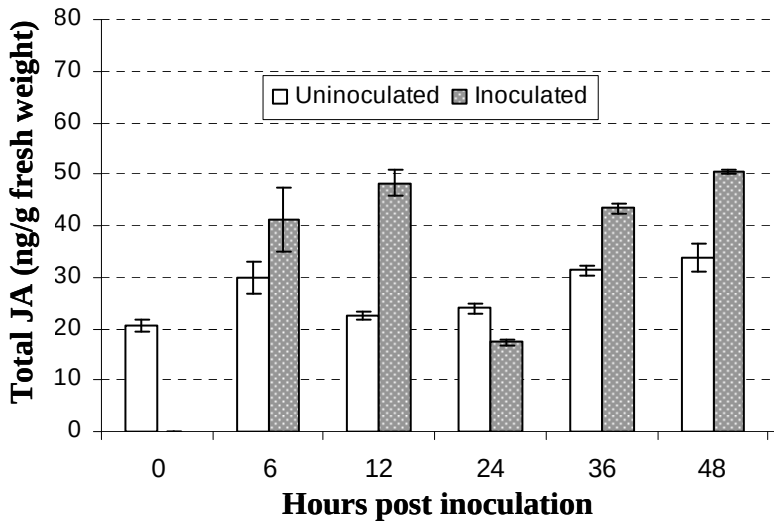

Total JA (ng/g fresh weight)
80
70
60
50
40
30
20
10
0
Uninoculated
Inoculated
0
6
12
24
36
48
Hours post inoculation

Figure 4

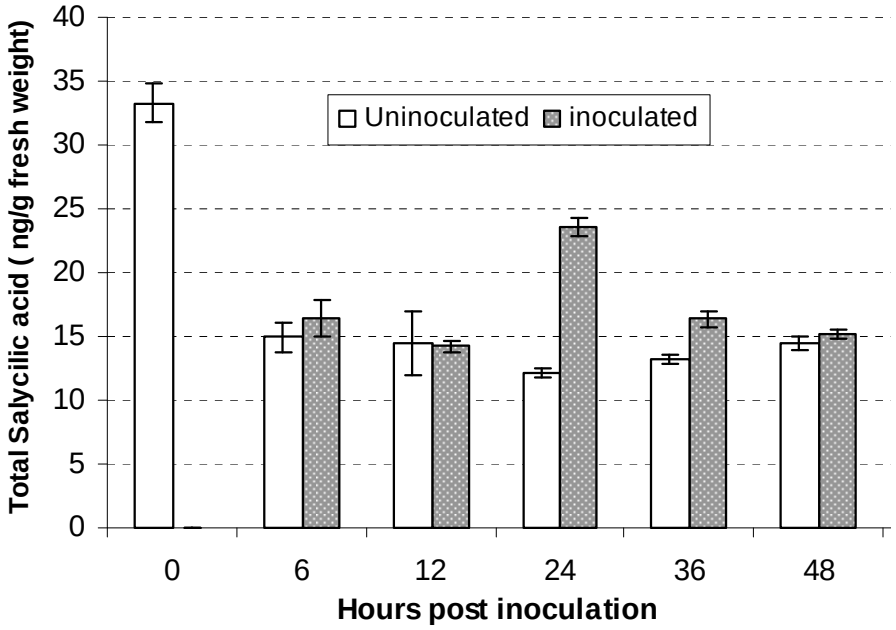

Total Salycilic acid (ng/g fresh weight)
40
35
30
25
20
15
10
5
0
Uninoculated
inoculated
0
6
12
24
36
48
Hours post inoculation

Figure 5

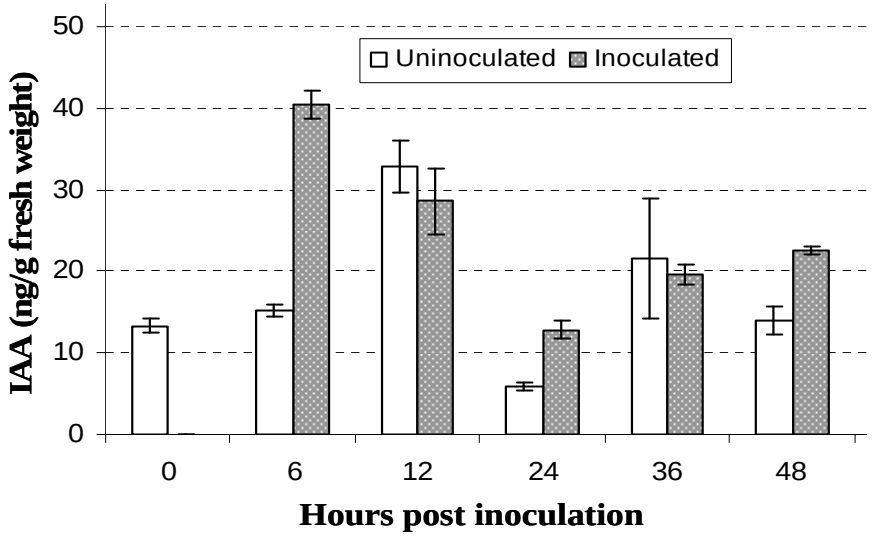

50
40
30
20
10
0
Uninoculated
Inoculated
IAA (ng/g fresh weight)
0
6
12
24
36
48
Hours post inoculation

Figure 6

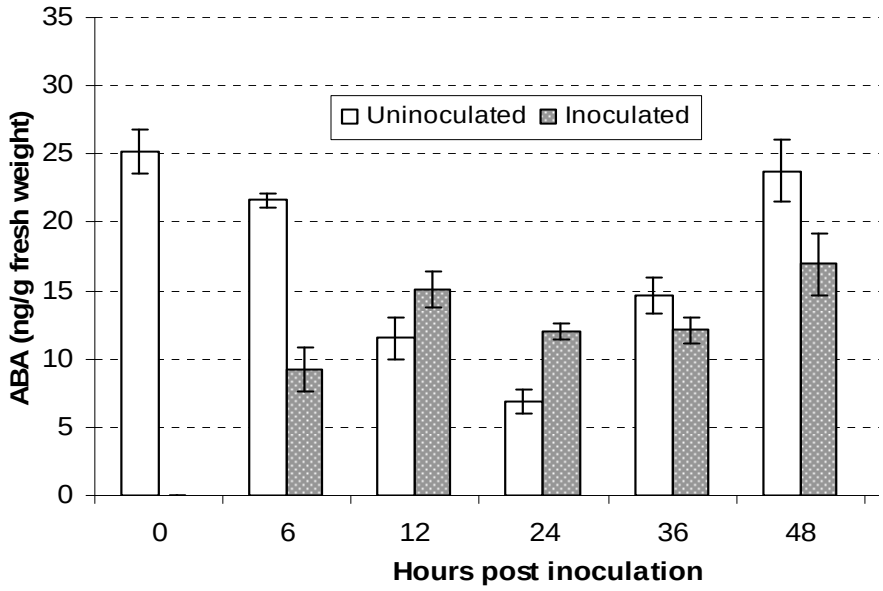
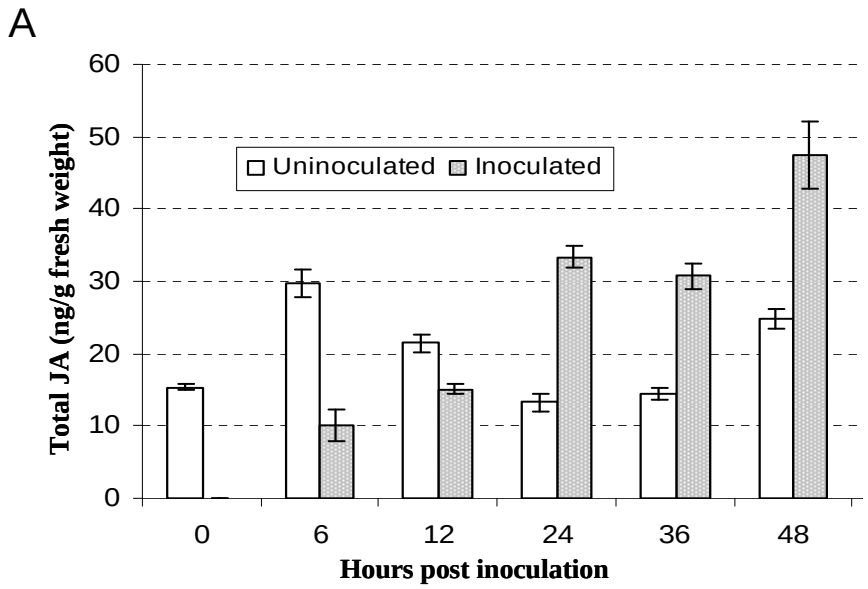

35
30
25
20
15
10
5
0
ABA (ng/g fresh weight)
Uninoculated
Inoculated
0
6
12
24
36
48
Hours post inoculation

Figure 7.


A
60
50
40
30
20
10
0
Total JA (ng/g fresh weight)
Uninoculated
Inoculated
0
6
12
24
36
48
Hours post inoculation

B

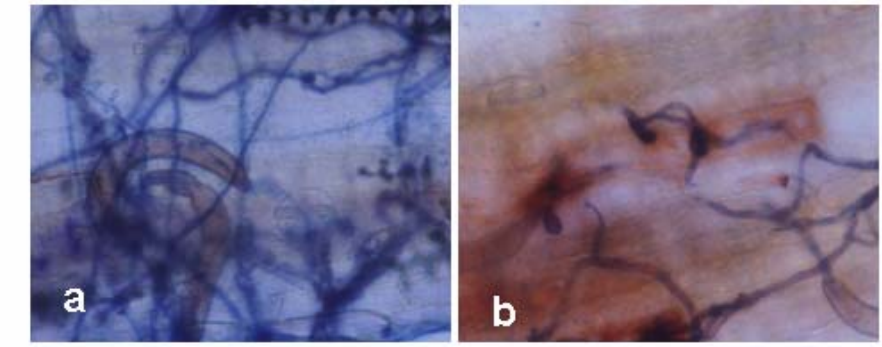

a
b

Table 1. Summary of the putative *cis* regulatory elements in the *y1* promoter region

cis element	Sequence	Start position	strand	function
ABRE	TACGTG	-1288	+	ABA responsive
ABRE	TGCCGT	-768	+	ABA responsive
ABRE	CCCACG	-199	+	ABA responsive
Motif II b	CCGCCCCGCT	-147	+	ABA responsive
EIRE	TTCGACC	-500	+	Elicitor responsive element as that present in PR1 promoter
TATA	TATATTATG	-31	+	binding RNA poly II
HSE	AAAAAATTTC	-1972	+	Heat stress responsive ness
HSE	AGAAAATTCG	-1292	+	Heat stress responsive ness
W box1	TGACC/T	-491	+	Binding WRKY proteins
W box2	TGACC/T	-779	+	Binding WRKY proteins
W box3	TGACC/T	-872	+	Binding WRKY proteins
W box5	TGACC/T	-1767	+	Binding WRKY proteins
W box6	TGACC/T	-1795	-	Binding WRKY proteins
TC rich repeat	ATTTTCTTCA	-1297	-	cis-acting element involved in defense and stress responsiveness
MYS	TAACTG	-1146	-	MYB binding site involved in drought-inducibility
MYS	CGGTCA	-777	-	MYB Binding Site
TGA element	TGACG	-1905	-	cis-acting regulatory element involved in the MeJA-responsiveness
CGTCA motif	CGTCA	-1909	+	cis-acting regulatory element involved in the MeJA-responsiveness
WUN motif	TCATTACGAA	-1524	-	wound-responsive element

Chapter 5

Summary and perspective

Phytoalexins are low molecular weight compounds produced *de novo* in plants in response to infection or stress (Purkayastha, 1994; Van Etten et al., 1994; Hammerschmidt, 1999). A very large number of phytoalexins belonging to diverse chemical classes have been identified (Knogge et al., 1987; Snyder and Nicholson, 1990; Kodama et al., 1992; Paxton, 1995; Hammerschmidt, 1999). The biosynthetic pathways that give rise to these compounds have been established in some model plant systems (Purkayastha, 1994) and are still under investigating in many others. In sorghum, the challenges by pathogenic or non pathogenic fungi induce the biosynthesis of phytoalexins that belong to a flavonoid class called 3-deoxyanthocyanidin (Nicholson et al., 1987; Hipskind et al., 1990; Snyder and Nicholson, 1990; Lo et al., 1996). These compounds appear as red-brown pigments at the primary infection sites in response to fungal attack. The nature of these compounds and their similarity in chemical structure to flavan-4-ols, the precursor of phlobaphene pigments that appear in sorghum floral tissues and mature leaves, have suggested that sorghum 3-deoxyanthocyanidins and flavan-4-ols may be synthesized via a common or overlapping pathway (Kambal and Bate-Smith, 1976; Stich and Forkmann, 1988; Styles and Ceska, 1989; Schutt and Netzly, 1991; Grotewold et al., 1998; Wharton and Nicholson, 2000; Chopra et al., 2002; Winefield et al., 2005). A large body of evidence has demonstrated that an R2R3 myb transcription factor encoded by a

gene called *yellow seed1* (*y1*) is required for the biosynthesis of flavan-4-ols in sorghum (Chopra et al., 1999; Boddu et al., 2005; Boddu et al., 2006). To gain better understanding of genetics of sorghum-fungus interactions and of 3-deoxyanthocyanidin biosynthesis, I have studied the following objectives:

The first objective of my Ph.D. work was focused on investigating the possible role of *y1* in controlling the biosynthesis of sorghum 3-deoxyanthocyanidins phytoalexins. We used a transposon-based approach to generate two near isogenic sorghum lines that differ in the functionality of the *y1* locus. The candystripe sorghum stock that carries an active transposable element, *candystripe1* (*cs1*) in the second intron of the *y1* gene was used for generation of these lines. We have used the phlobaphene pigmentation phenotype in the pericarp as a marker to identify several transposition excisions of the *cs1* element out of *y1*. The phenotypes of the resulting lines were both fully red or white pericarps and glumes. The molecular characterization of red pericarp events indicated that these phenotypes are due to the complete excision of the *cs1* transposon from *y1*. A 2-bp foontprint was found at the site of excision. Analysis of the *y1* structure in white pericarp events was done using Southern blot and PCR analysis which demonstrated the presence of an internal deletion in the *y1* gene. The white pericarp phenotype showed complete genetic stability in subsequent generations. Both the red and white pericarp events were genetically tested and found to be allelic to the *y1* gene and were designated as *Y1-rr* (red pericarp and red glume) and *y1-ww* (white pericarp and white glume), respectively.

We compared the phytoalexin biosynthetic ability of these lines in response to pathogenic and non pathogenic fungi. Biochemical analysis indicated that the sorghum line that has a functional *y1* (*Y1-rr*) synthesized all the known 3-deoxyanthocyanidins. In contrast, the sorghum line that carried a non-functional *y1* (*y1-ww*) was defective in this trait. Gene expression analysis in sorghum seedlings after infection indicated that the transcription of both *y1* and its target genes in the flavonoid pathway were up-regulated in *Y1-rr* seedlings. Transcription of *y1* preceded that of the other genes and showed induction as early as 3 hpi. Steady state transcripts of *y1* or its target genes were not detected in the *y1-ww* line indicating that the transcription of these flavonoid genes is *y1* dependant in fungal challenge tissues. These results give direct genetic evidence that *y1* is required for the biosynthesis of sorghum 3-deoxyanthocyanidins and may regulate the transcription of flavonoid biosynthetic genes.

The near isogenic lines used in this study provided valuable genetic materials for studying the potential contribution of 3-deoxyanthicyanidins phytoalexin to sorghum resistance against anthracnose disease. Disease analysis using these near isogenic lines and sorghum anthracnose fungus, *Colletotrichum sublineolum,* demonstrated that the sorghum line that carries a functional *y1* (*Y1-rr*) contained the fungal infection at the primary infection site. This was indicated by the localized red spots on the infected leaves. On the other hand, the *y1-ww* line was deficient in biosynthesis of 3-deoxyanthocyanidins and exhibited symptoms typical of anthracnose disease indicated by the black spots representing fungal fruiting bodies. These results provided genetic

evidence that *y1* and 3-deoxyanthocyanidins are required for sorghum resistance against anthracnose disease.

Sorghum and maize are related and have been suggested to arise from a common progenitor about 16 millions years ago (Doebly et al., 1990; Bennetzen and Freeling, 1997; Gaut et al., 2000). Regardless of the high level of genome synteny between sorghum and maize (Melake-Berhan et al., 1993; Devos and Gale, 2000), the flavonoid pathways of these two species exhibit two major differences: First, sorghum accumulates flavan-4-ols and phlobaphenes in floral as well as in leaves; however, these compounds are synthesized in maize floral tissue only. Second, unlike sorghum, maize plants do not synthesize 3-deoxyanthocyanidins in response to fungal attack (Hipskind et al., 1996a). In maize, the biosynthesis of flavan-4-ols occurs under the control of a *myb* transcription factor *pericarp color1* (*p1*). *p1* and *y1* are orthologues with a high degree of similarity in their coding sequences but very poor similarity in the regulatory regions (Boddu et al., 2006). These two genes have almost the same expression patteren except that *y1* has significant expression in leaf and is induced by fungal infection. Therefore, the second objective of the current work was to test the ability of the sorghum *y1* gene to induce flavan-4-ols, 3-deoxyanthocyanidins, and other defense related flavonoid and phenolic compounds in transgenic maize leaves.

We developed transgenic maize lines expressing either an *Y1::GUS* fusion or the intact *y1* gene and used these plants to test the hypothesis that the expression of *y1* in leaves and its induction by the fungus is a property of the *y1* promoter. Analysis using the *Y1::GUS* construct indicated that the *y1* promoter is constitutively expressed in maize

floral and vegetative tissues and is responsive to fungal infection. Interestingly, *y1* induced phlobaphene pigmentation in maize floral tissues and these phenotypes were stably inherited through generations. In addition, *y1* drives the flavonoid pathway in maize leaves towards the biosynthesis of flavan-4-ols, and enriched maize silks and leaves with a variety of defense related phenylpropanoid compounds. Further, *y1* induced the accumulation of many c-glycosyl flavones whose insecticidal activity against *Helicoverpa zea* has been demonstrated and attributed to the ability of these compounds to disrupt the amino acid metabolism in the insect gut (Waiss et al., 1979; Snook et al., 1994; Byrne et al., 1996). Furthermore, the accumulation of these defense related compounds in transgenic maize leaves resulted in enhanced resistance against corn southern leaf blight. The results from the heterologus expression of *y1* in maize suggest that possible evolutionary modifications in *y1* and *p1* promoters might account for the flexibility of the flavonoid pathway to produce different but related end products in sorghum and maize.

The third objective of my research was aimed at understanding the signaling cascades upstream the biosynthesis of 3-deoxyanthocyanidins phytoalexins in sorghum. As mentioned above, these phytoalexin compounds accumulate early on at the primary infection sites indicating the presence of an active defense system involving coordination of signaling pathways with multiple components. This system includes the rapid recognition of the pathogen, transmission of the generated signal(s), and production of these defense compounds in the sorghum cells under attack. The biosynthesis of sorghum 3-deoxyanthocyanidins involves activation of the flavonoid pathway which occurs under

the control of a myb transcription factor encoded by *yellow seed1* gene (*y1*; Chapter 2). Therefore, *y1* is an important component in the signaling cascade that leads to the biosynthesis of these compounds. However, the upstream signal that leads to its activation is not known. Phytohormones have been demonstrated in many biological systems to be crucial components of the signaling cascades that regulate the biosynthesis of defense related compounds (Glazebrook, 2001; Thomma et al., 2001; Gachomo et al., 2003; De Vos et al., 2005; Pieterse and Dicke, 2007; Kunkel and Brooks, 2002).To get an insight into the phytohormones that may play a role, we first dissected the *y1* promoter using Plant Care database(http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

We found two *cis* recognition sequences similar to those identified in JA-responsive genes. In addition, we found that the *y1* promoter is enriched with six W boxes similar to those identified in the promoter of other defense related proteins (Eulgem, 2005). On the other hand, we did not find any "*as-1*" element that has been shown to be the target of SA-responsive genes (Rushton and Somssich, 1998; Garreton et al., 2002). Consistent with these results, our phytohormonal analysis indicated that the biosynthesis of sorghum 3-deoxyanthocyanidin phytoalexins was associated with early accumulation of JA and a limited change in SA.

Based on the current and previously available results, we propose the following model for sorghum phytoalexin biosynthesis. First, sorghum cells recognize the fungus or fungal elicitor via a LLR type of protein (Hipskind et al., 1996b). Second, such recognition generates signal(s) that leads to induction JA biosynthesis (Creelman and Mullet, 1997). Third, the fungus-induced JA activates expression of one or more specific

JA-induced WRKY protein(s) (Salzman et al., 2005). Fourth, these proteins recognize one or more W boxes in the *y1* promoter and turn on its expression. Once *y1* is activated, it trans-activates the expression of the flavonoid structural genes and drives the sorghum flavonoid pathway toward 3-deoxyanthocyanidins' biosynthesis.

In summary, research conducted during my tenure as a Ph.D student contributed significantly to our knowledge about the mechanisms controlling the biosynthesis of sorghum 3-deoxyanthocyanidin phytoalexins. In addition, it provided insights into how evolutionary changes in the regulatory regions of transcription factors could shape the metabolic pathways of plants to cope with the continuously changing environmental conditions. Dissection of the *y1* promoter at the molecular level will be informative and may help to directly assign a definite role of the *cis* recognition sequences identified in the current study during fungal recognition. This might also lead to the identification of other components that works upstream of *y1* in response to fungal infection. In addition, investigating the impact of the heterologus expression of *y1* on the primary plant metabolism will be very interesting because of the close link between the flavonoid and phenylpropanoid pathways to the shikimic acid pathway, one major link between primary and secondary metabolism. Further, one can take advantage of maize genetic and genomics resources and perform microarray analysis to identify and investigate other genes that might work coordinately with *y1* in maize.

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