NITROGEN-USE EFFICIENCY AND GENE EXPRESSION PROFILING OF TROPICAL MAIZE HYBRIDS SELECTED FOR CONTRASTING RESPONSES TO NITROGEN FERTILIZER

BY

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ABSTRACT

Low soil Nitrogen (N) and sub-optimal N fertilizer application result in low Grain Yield (GY) in maize. Genotypes with improved N-Use Efficiency (NUE) are beneficial to low-input agriculture. To facilitate the genetic improvement of tropical maize for NUE, information is required on the relative importance of N-Uptake Efficiency (NUpE) and N-Utilization Efficiency (NUtE), both components of NUE, the relationships among the gene networks in the developing cob tissue and agronomic traits under limited N. This study was undertaken to evaluate genetic variation for NUE and investigate gene responses in the developing maize cob under sub-optimal N fertilizer application.

Fourteen tropical maize hybrids differing in GY under low-N conditions were grown at no-N (0 kg N/ha), low-N (30 kg N/ha), and high-N (90 kg N/ha) in a randomized complete block design with four replications from 2006 to 2008. Data collected on GY and its components, NUE, NUpE, NUtE, and N-related traits were analysed using ANOVA at p=0.05. Path analysis was carried out to capture the intricate relationships among traits related to GY and NUE. Microarray technique using Maize Oligonucleotide array slides containing ~57000 probes were used to identify differentially expressed genes in developing cob tissues harvested from three N-use efficient hybrids compared to an N-use inefficient reference hybrid. Differentially expressed genes were validated by quantitative Real Time Polymerase Chain Reaction (qRT-PCR).

Genotype and Genotype × Nitrogen interaction were significant for GY and NUE-related traits. Mean GY across years was reduced by 76.5% at no-N and 35.4% at low-N. It varied from 2.0 to 3.2 Mg/ha at low-N and 3.3 to 4.4 Mg/ha at high-N. Number of kernels (KN) was the GY component most severely reduced under nitrogen stress. Nitrogen use efficiency, NUtE and NUpE increased by 61.4, 42.1 and 21.0% respectively from high-N to low-N. Grain yield was positively and significantly correlated with NUE, NUtE and NUpE at both low-N and high-N. Stover nitrogen content at silking and NUE had strong positive direct effects on GY under low-N and high-N. Both NUtE and NUpE had significant positive direct effects on NUE.

The relative importance of NUpE and NUtE varied depending on genotype and environment. Four hybrids (4001/4008, KU1409/9613, KU1409/4008 and 4008/1808) produced similar above average GY at low-N and high-N but differed in their NUpE and NUtE. Gene expression profiling of developing cob tissues revealed that at low-N, 163 of the probes on the array showed differential expression across the test hybrids. Under low-N, the probe MZ00019244 corresponding to the L-asparaginase gene showed the strongest relative mRNA expression in the highest yielding hybrid (KU1409/9613). L-asparaginase mRNA expression level was positively and significantly correlated with GY, KN and NUtE.

Genetic variation for nitrogen use efficiency and its components was present among the tropical hybrids. L-asparaginase mRNA expression would be a valuable tool for selecting maize genotypes with high nitrogen utilization efficiency and superior grain yield under low soil nitrogen.

Keywords: Maize grain yield, N-use efficiency, N-uptake efficiency, N-utilization efficiency, L-asparaginase gene.

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CERTIFICATION BY SUPERVISORS

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DEDICATION

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CHAPTER 1
INTRODUCTION

Maize, *Zea mays* L., is the most widely cultivated crop and most important food staple in sub-Saharan Africa, where it accounts for up to 70% of the daily human calorie intake (Martin *et al*., 2000; FAO, 2007). In West and Central Africa, not only has maize replaced sorghum and millet as food staple, it is a source of cash for small-holder farmers (Smith *et al*., 1997; Fakorede *et al*., 2003). The current average yields for maize grain from the major maize production ecologies of sub-Saharan Africa are significantly lower than those obtained in most other areas of the world. For example, Pingali and Pandey (2001) reported a yield potential of 5.0 Mg/ha in tropical highlands, 4.5 Mg/ha in tropical lowlands and 7.0 Mg/ha in subtropical and mid-altitude zones, with yields in farmers fields of 0.6, 0.7 and 2.5 Mg/ha, respectively. Current average grain maize yield across sub-Saharan Africa is 1.78 Mg/ha (FAO, 2009).

As in other regions of the world where green revolution occurred, Africa's anticipated green revolution is expected to be grain-based, and the most likely crop for this in West and Central Africa is maize (Badu-Apraku *et al*., 2009). Despite the great potential of maize in the sub-region, its current level of production will not adequately meet the demand by the rising population in the sub-region (Bänziger *et al*., 2004). Several biotic and abiotic factors constrain maize productivity in West and Central Africa. The soils of the major maize producing ecologies in the sub-region are inherently low in nitrogen (N), making N deficiency a common feature in maize production (Jones and Wild, 1975).

One important cultural practice that accounts for over 40% of grain yield increases in maize is the increased use of supplemental N fertilizer (Sinclair and Muchow, 1995; Smil, 2002). Since maize grain yield response to fertilizer N application in N-deficient soils is high (Moose and Below, 2008), an estimated 5 MMg of N fertilizers are applied annually to maize production in the industrialized countries, and its use is on the rise in developing countries (FAO, 2004). There is, however, a growing concern on the effects of excessive use of fertilizer on production costs, as

well as on environmental and public health (McNight *et al*., 1999), as it does not only increase crop input and energy costs, but also negatively impacts on the environment by altering soil, water and air quality (Tilman *et al*., 2002). Fertilizer use in Africa is reported to be low, with an average of 8 kg/ha of nutrients (IFDC, 2006; Heisey *et al*., 2007). The limited local supply of N fertilizers in Africa and inadequate transportation and distribution infrastructure contribute to as much as five-fold higher prices compared to global market prices (Mosier *et al*., 2005). The combination of these factors with the low purchasing power of the predominant resource-poor smallholder farmers in sub-Saharan Africa, either prevent the use, or reduce the quantity, of N fertilizer applied by resource-poor farmers (Bänziger *et al*., 1999; Crawford *et al*., 2005).

For a sustainable agriculture, farmers in developed and developing must optimize their agro-technical production systems to avoid pollution of the environment by reactive N species and reduce its associated health risks and costs (Dawson *et al*., 2008). Although the form and amount of N available to the plant can be managed by adopting improved cultural practices, the innate efficiency of the plant to utilize available soil N must be genetically addressed (Pathak *et al*., 2008). Maize varieties developed by breeding programs are highly productive and responsive to N application, but they usually exhibit low N-use efficiency (O'Neill *et al*., 2004). The development and adoption of maize varieties with improved N-use efficiency could give improved yields with less supplemental N, reduce input costs and limit the risk of N pollution to the environment (Presterl *et al*., 2002).

For grain maize, N-use efficiency is defined as the grain yield per unit N available from the soil, including fertilizer N (Moll *et al*., 1987). N-use efficiency is the product of two major components namely, N-uptake efficiency and N-utilization efficiency. N-uptake efficiency is the fraction of applied fertilizer N found in the plant at maturity, while N-utilization efficiency is the ratio of grain yield to plant N. Previous studies have reported the existence of genotypic differences in N-use efficiency (Ma *et al*., 1998; Muchow, 1998; Akintoye *et al*., 1999; Bertin and Gallais, 2000; Gallais and Hirel, 2004; Worku *et al*., 2007). Quantitative trait loci (QTLs) have also been detected for N-use efficiency at various levels of N fertilizer application (Agrama *et al*., 1999; Bertin and Gallais, 2001). These suggest that many genes and combination of genes are differentially expressed according to the amount of N provided to the plant (Bertin and Gallais, 2000; 2001). Breeding for improved N-use

efficiency is therefore considered feasible (Presterl *et al*., 2003) both by marker-assisted selection and genetic engineering (Hirel *et al*., 2007a).

In order to design breeding and crop management strategies to improve plant N-use efficiency, a better understanding of the major processes associated with N-use efficiency, such as N-uptake and N-utilization efficiency is required (Uribelarrea *et al*., 2007). Conflicting results on the relative importance of these two main components for improving N-use efficiency are reported in the literature. Under low-N conditions, Moll *et al*. (1982), Ma *et al*. (1998) as well as Bertin and Gallais (2000) reported that N-utilization efficiency is more important than N-uptake efficiency. On the other hand, Kamprath *et al*. (1982) reported that N-uptake is more important than N-utilization. Other studies, Worku *et al*. (2007) for tropical maize, Weisler *et al*. (2001) for temperate maize and Ortiz-Monasterio *et al*. (2001) for wheat have reported that both N-uptake and N-utilization are important for optimal performance under low-N condition. In addition to these divergent positions, research findings have not been conclusive on the regulatory mechanisms and genes that control these traits in maize. Consequently, an increased knowledge of the regulatory mechanisms and genes involved in plant N economy in maize is required (Hirel *et al*., 2007c). A combination of genetic and physiological information coupled with the optimization of agronomic practices can provide a more complete model of genotype-to-phenotype relationships and genotype-by-environment interaction for N-use efficiency (Good *et al*., 2004; Edmeades *et al*., 2004; Hirel *et al*., 2007b). Thus, a functional genomics approach that combines genetic and physiological studies of whole plant N responses is considered most appropriate for elucidating the gene networks regulating N-use efficiency.

Functional genomics approaches allow comprehensive studies of cellular metabolisms in specialized tissues of whole organisms (Dixon, 2001). It concentrates on how genes function alone and in networks (Edmeades *et al*., 2004). Genomic approaches involving transcriptome, proteome and metabolome profiling have been applied to investigate plant responses to abiotic and biotic stresses. For example, studies in maize (Yu and Setter, 2003; Riccardi *et al*., 2004), rice (Hazen *et al*., 2005), sorghum (Pratt *et al*., 2005), sugar beet (Hajheidari *et al*., 2005) as well as the model plant *Arabidopsis thaliana* (Kawaguchi *et al*., 2004) have employed genomics approaches to elicit understanding of the functional bases of drought tolerance. Functional genomics approaches are also being exploited to elucidate plant-parasitic weed interactions and resistance (Castillejo *et al*., 2004; Die *et al*., 2009). Similarly,

several transcriptomic approaches on *Arabidopsis* (Wang *et al*., 2004; Scheible *et al*., 2004), *Solanum lycopersicum* (tomato) (Wang *et al*., 2001), and *Oryza sativa* (rice) (Wang *et al*., 2002), have been used to identify more than 1000 genes differentially expressed upon nitrate supply (Gutierrez *et al*., 2007).

While most studies directed at elucidating the genetic and physiological basis of N-use efficiency in maize and other plant species have concentrated on vegetative tissues such as the leaves and roots (Hirel *et al*., 2007c), only few have highlighted the importance of reproductive sink organs such as the developing ear in maize (Seebauer *et al*., 2004; Cañas *et al*., 2009) and the spike in wheat (Howart *et al*., 2008) to N-use efficiency. The objectives of this study therefore were to: (i) evaluate genetic variation for N-use efficiency among selected tropical maize hybrids, (ii) establish the relative importance of N-uptake efficiency and N-utilization efficiency to N-use efficiency, (iii) determine agronomic and physiological traits that could be used to optimize selection for high grain yield under both N-stress and non N-stress conditions, (iv) identify genes associated with N responses in developing earshoots of selected tropical maize hybrids, and (v) identify those genes that are promising for marker assisted-selection and breeding for efficient use of N.

CHAPTER 2
LITERATURE REVIEW

2.1 Maize Production in sub-Saharan Africa

Maize (*Zea mays* L.) is a cereal crop well adapted to agro-ecological zones with mono-modal rainfall distribution and 120 to 180 day growing period (Carsky and Iwuafor, 1999). It is the most important cereal crop in sub-Saharan Africa with an estimated production of 50.6 MMg from 28.5 million hectares (FAO, 2009). Maize cultivation in West and Central Africa has spread from its traditional rainforest into the savannas, replacing other cereal crops such as sorghum and millet, most especially in areas with good access to fertilizer inputs and markets (Smith *et al*., 1997; Fakorede *et al*., 2003) and accounts for an estimated 34% of the total cereal production in the sub-region (FAO, 2009).

Maize is an important source of calories in many African countries. In sub-Saharan Africa, the average daily calorie intake of maize ranges from 15 – 50% (FAO, 2007). Over the last three decades, maize production in Africa increased at a mean annual rate of about 3%, with current average yield of about 1.87 Mg/ha (FAO, 2008). During the same period, in West and Central Africa, total maize production increased from about 3.59 to 17.98 MMg per year, with yield per hectare from 0.84 to 1.56 Mg and acreage from 4.28 to 11.51 million hectares (FAO, 2008). The average grain maize yield in sub-Saharan Africa has remained low (1 – 2 Mg/ha) (FAO, 2008) despite the availability of improved varieties and agronomic practices. Thus, maize production has not kept pace with consumption, and about 10 MMg of maize are imported annually in Africa (Cassman, 2007).

The spread of maize cultivation into the savanna of West and Central Africa has enhanced its production and productivity. The Guinea savanna has been identified as the most suitable for maize production because of its more favourable growing conditions compared with the traditional forest zone (Kassam *et al*. 1975; Kim *et al.,* 1986). Average on-farm yield of maize in the West African savanna has increased from less than 1.0 Mg/ha in the 1970s to more than 2.5 Mg/ha (Fakorede *et al*., 2003; Ortiz and Hartmann, 2003). The gap between potential and national maize grain yield

in the West African savanna are mainly due to abiotic and biotic stresses (Kamara *et al*., 2004). Over the last 40 years, more than 200 maize varieties comprising hybrids and open-pollinated varieties, targeted at specific agro-ecologies, have been released following the combined effort of the International Institute of Tropical Agriculture (IITA) and the West and Central African Maize Network (WECAMAN) (Manyong *et al*., 2003).

In Nigeria, maize is grown throughout the country from the high rainfall forest area of the southeast to the low rainfall Sudan savanna of the north. Nigeria produces about 58% and 15% of the maize grown in West Africa and Africa, respectively (FAO, 2007). It is most widely grown in the northern Guinea savanna where it is one of the two major crops on 30 – 40% of the area under agricultural production (Fakorede *et al*., 1993; Smith *et al*., 1997). There has been a rapid increase in maize production and consumption in Nigeria. Although the successful development of high yielding varieties and improved crop management systems has contributed to the improved production and consumption of maize (Smith *et al*., 1997), these increases have resulted largely from extensive rather than intensive cultivation (Byerlee and Eicher, 1997; Fakorede *et al*., 2001).

Nigeria's annual maize production in 2008 was estimated at about 7.53 MMg from a land area of about 3.85 million hectares, with per hectare yield of 1.96 Mg (FAO, 2008). Maize grain yield per hectare varies from one agro-ecological zone to the other. Under mono-cropping and good management conditions in research stations, grain yields of 3 to 5 Mg/ha and 4 to 6 Mg/ha could be achieved from improved varieties in the forest and savanna agro-ecologies respectively (Carsky and Kling, 1997). Maize grain yield of 1.96 Mg/ha in Nigeria is also low compared to the world average of 5.13 Mg/ha (Fajemisin, 1986; FAO, 2008) because most of the maize is produced under mixed cropping accompanied by poor nutrient supply, weed problem, low plant density, late planting and late first weeding (Carsky and Kling, 1997). In Nigeria and many countries of sub-Saharan Africa, a substantial fraction of the field maize grown with early rains is harvested and sold fresh as green maize. Green maize is physiologically immature maize harvested 21 – 22 days after mid-silking. This contrasts with developed countries where human consumption is mainly of sweet and super-sweet corn (Osayintola *et al*., 1992; Lee *et al*., 1999; Zan and Brewbaker, 1999). According to FAO statistics (FAO, 2008), green maize production in Nigeria in 2008 was about 0.579 MMg from a land area of 0.163 million hectares with average yield of

3.56 Mg/ha.

2.2 Maize productivity and Nitrogen fertilization

Nitrogen is a major requirement for the production of seeds (Mengel *et al*., 2006) and forage (Kingston-Smith *et al*., 2006). It is a vital plant nutrient and a major yield determining factor required for maize production (Adediran and Banjoko, 1995; Shanti *et al*., 1997). Nitrogen is often the most limiting nutrient in agro-ecosystems and is therefore applied in the highest quantities (Havlin *et al*., 1999; FAO, 2001). Its deficiency is one of the major limitations to maize productivity in world agriculture (Thomason *et al*., 2002). This is particularly so in the savannas of West and Central Africa where soils are mainly kaolinitic alfisols, low in organic matter and cation-exchange capacity (Jones and Wild, 1975) and where the use of inorganic fertilizers is low (Kamara *et al*., 2005). Maize has a high and relatively rapid nutrient requirement (Carsky and Iwuafor, 1999). It requires high amount of N for high yields and every megagramme of grain produced removes approximately 20 – 25 kg of N from the soil (Muzilli and Oliveira, 1992 cited by Sangoi *et al*., 2001). However, African soils cannot supply the quantities of nutrients required. Loss in maize grain yield due to low N stress alone varies from 10 – 50% (Wolfe *et al*., 1988; Logrono and Lothrop, 1997), resulting in a loss in income worth billions of dollars.

All phases of maize growth are affected by variation in N supply, including the development, activity, and senescence of leaves, and the initiation, growth and composition of ovules (Muchow, 1988; Uhart and Andrade, 1995a, 1995b). The physiological processes influenced by nitrogen include (i) establishment of the plant's photosynthetic capacity (Hageman and Below, 1984; Sallah *et al*., 1998); (ii) prolonging effective leaf area duration and delaying senescence (Earl and Tollenaar, 1997); (iii) increasing the plant's rooting depth (Nielsen and Halvorson, 1991); (iv) ear and kernel initiation, contributing to define maize sink capacity (Tollenaar *et al*., 1997); and (v) helping to maintain functional kernels throughout grain filling, leading to a greater number of developed kernels and increased final kernel size (Huber *et al*., 1994; Jones *et al*., 1996). The availability of N also affects a number of agronomic traits. Its deficiency has been reported to delay silking (Russel, 1991), decreases pre-anthesis crop growth rate (McCollough *et al*., 1994), dwindles leaf area index at flowering and accelerates leaf senescence rates throughout the life cycle (Wolfe *et al*., 1988). Thus, for optimum growth, it is essential that it is made available in sufficient

quantities throughout the growing season (Kogbe and Adediran, 2003).

The amount of fertilizer to be applied depends mainly on two factors, namely: the projected yield that is attainable in that locality and the fertility level of the soil as determined by soil test (Onwueme and Sinha, 1991). Balasubramanian *et al*. (1978) as well as Chude *et al*. (1994) conducted a series of response trials leading to a recommendation of 100 – 120 kg N/ha, 60 kg P_2O_5/ha and 60 kg K_2O/ha for sub-humid Nigeria. Studies conducted by Akintunde *et al*. (1993) obtained an optimum N rate of 60 kg N/ha for the forest zone and 120 kg N/ha for the northern and southern Guinea savanna. Agboola (1968) as well as Ologunde and Ogunlela (1984) obtained similar results. Inorganic fertilizer use in countries of West and Central Africa is very low. Resource poor farmers find it too expensive and therefore apply nitrogen fertilizers at sub-optimal levels (McCown *et al*., 1992). FAO (1992) reported that the average rate of fertilizer use in Nigeria is about 12 kg nutrients per hectare of arable land, and figures for other West African countries are lower. Manyong *et al*. (2001) estimated the average N fertilizer application rate to be 40 kg ha^{-1} for two benchmark villages in the northern guinea savanna of Nigeria, which was far lower than the recommended dose of 120 kg N/ha.

The ultimate fate of nitrogen absorbed by the maize plant is to make growth and development of all stages of the life cycle of the plant possible and to convert much of the reduced N to grain protein. Genotypic differences exist in the capacities of maize varieties for N uptake, assimilation and redistribution of N from vegetative to reproductive tissues (Pollmer *et al*., 1979; Beauchamp *et al*., 1976). Kanampiu *et al*. (1997) reported that one way of increasing N-use efficiency is to avoid excessive application of N fertilizers. Application methods that avoid applying large amounts of N at one time can also increase N-use efficiency (Wuest and Cassman, 1992). In order to ensure adequate supply of added N at critical stages of plant growth and reduce N leaching losses due to heavy rainfall, Balasubramanian *et al*. (1978) reported that the total N fertilizer should be split into two equal doses and applied first at planting and later at 4 – 6 weeks after planting. They also suggested that top dressing of urea should not be delayed beyond 6 weeks after planting to avoid yield reduction. According to Mullen *et al*. (2003), multiple timely inputs of N during the growing season, while potentially costly, could significantly increase N-use efficiency.

2.3 Nitrogen fertilization, the environment and sustainable agriculture

Globally, fertilizer-N application accounts for about half of all N reaching croplands (Fixen and West, 2002) leading to the generation of excess fixed nitrogen. Excess fixed nitrogen, in various guises, augments the greenhouse effect, diminishes stratospheric ozone, promotes smog, contaminates drinking water, acidifies rain, eutrophies bays and estuaries and stresses ecosystems (Socolow, 1999). These effects result from the emission of nitrogen oxides which reacts with the stratospheric ozone, and the emission of ammonia into the atmosphere (Ramos, 1996; Stulen *et al*., 1998) when high rates of N fertilizers are applied to agricultural fields (Tilman 1999). According to FAO, about 82 MMg of nitrogen fertilizers were applied globally in 2001 (FAO, 2001), up from only 1.3 MMg in 1930 and 10.2 MMg in 1960 (Frink *et al*., 1999). Nitrogen fertilizer use is predicted to further increase to 240 MMg by 2050 (Tilman, 1999). It is estimated that 50 – 70% of applied N is lost from the soil-plant system (Peoples *et al*., 1995). Cereal production accounts for about 60% of the total N used (FAO, 1995) with estimated world N-use efficiency for cereals ranging from 25–50% (Raun and Johnson 1999; Tilman *et al*., 2002). These figures suggest that at least 50% of applied nitrogen is lost from agricultural systems.

There are many causes or pathways for nitrogen loss from the soil-plant system. These include losses via denitrification (Burford and Bremmer, 1975; Olson *et al*., 1979; Burkart and James, 1999), runoff (Gascho *et al*., 1998; Burkart and James, 1999), and leaching (Goss and Goorahoo, 1995; Paramasivam and Alva, 1997). Fertilizer N losses via denitrification have been estimated at 9.5% in winter wheat (Auklakh *et al*., 1982), 10% in rice (DeDatta *et al*., 1991), and >10% in corn (Hilton *et al*., 1994). Blevins *et al*. (1996) as well as Chichester and Richardson (1992) reported that fertilizer N losses due to surface runoff ranged between 1 and 13%. Urea fertilizers applied to the surface without incorporation can result in ammonia (NH_3) volatilization losses in excess of 40% (Fowler and Brydon, 1989; Hargrove *et al*., 1977). Nitrate N loss through tile drainage in cooler temperate climates is about 26 kg N/ha/yr or 23% of total N applied (Drury *et al*., 1996). Average annual nutrient loss per hectare for sub-saharan Africa rose from 22 kg N, 2.5 kg P and 15 kg K in 1982 to 1984 to 26 kg N, 3 kg P and 19 kg K in 2000 (Stoorvogel *et al*., 1993). Although there are many causes and pathways for N loss, application of N in amounts that exceed crop requirements elevates post harvest nitrate-nitrogen (NO_3-N) levels in the soil and increases the potential for leaching into ground water supplies (Schepers *et al*., 1991;

Raun and Johnson, 1995). All these losses can have deleterious environmental effects or consequences (Vitousek *et al*., 1997; Glass, 2003) and therefore calls for the exploration of low input (Raven *et al*., 2005) and more precise (Day, 2005) agriculture.

According to FAO, rice, wheat, maize, and to a lesser extent, barley, coarse grain legumes, along with root crops are the most important crops cultivated in the world and account for the majority of the end-products used for human diets. These crops are likely to still contribute greatly to human nutrition in the next century (Hirel *et al*., 2007c). The high yields of rice, wheat, and maize largely contributed to the total increase in the global supply of food production since 1967 (Cassman, 1999) and this has been associated with a 7 – fold increase in the use of N fertilizers (Hirel *et al*., 2007c).

Sustainable agriculture has been defined as practices that over long term periods enhances environmental quality and the resource base on which agriculture depends, provides for basic human food and fibre needs, is economically viable, and improves the quality of life for farmers and the society as a whole (White *et al*., 1994). The global human population is expected to increase to 10 billion by the year 2070 and feeding all these peoples will require a more efficient use of agricultural lands (Frink *et al*., 1999; Tilman, 1999) As the intensification of agricultural production has been accompanied by severe N contamination of ground and surface water, marine water and the atmosphere (Matson *et al*., 1997), the possibility of accommodating the needs of the expanding world population by developing a more productive agriculture, while at the same time preserving the quality of the environment will be the challenge of the next decades (Dyson, 1999; Fixen and West, 2002). The development of crops with enhanced nutrient uptake is one component that could help in the achievement of this goal (Good *et al*., 2004).

In view of the problems associated with fertilizer N usage, the development of improved nutrient management strategies has been the primary focus of agricultural research for over a decade (Gauer *et al*., 1992; Spellman *et al*., 1996; Mulla and Bhatti, 1997; Khosla and Alley, 1999; Khosla *et al*., 2002). Crop management strategies that improve nutrient use efficiency may increase farm profits and greatly reduce the deleterious environmental effects associated with fertilizer loss (Inman *et al*., 2005). According to Schlegel and Havlin (1995), the application of an economically optimal N rate minimizes NO_3 accumulation in the soil, thus lowering

the potential of NO_3 leaching into ground water. Improving the efficiency of absorption and utilization of soil applied N by maize cultivars should be an important goal of maize breeders (Rizzi *et al*., 1993). Improvement in N-use efficiency in crop plants may support the development of cropping systems that are more economically efficient and environmentally friendly (Agrama *et al*., 1999). Therefore, maize varieties with improved N-use efficiency, especially under low N conditions can contribute to sustainable agriculture (Presterl *et al*., 2002).

2.4 Nitrogen use efficiency: Definitions, components, and estimation

One of the most expensive nutrients to supply for crop production is nitrogen. Crop improvement strategies are therefore directed to the measurement and maximization of the efficiency of nutrient-use. Vitousek *et al*. (1997) advanced two reasons for the improvement of nutrient-use efficiency. First, the use of commercial fertilizers is one of the major costs associated with the production of high yielding crops. These costs are substantial for all producers and are often prohibitive for subsistence farmers. Second, the environmental damage associated with the use of N-based fertilizers is becoming significant. Matson *et al*. (2002) alerted of the significant consequences of the globalization of N deposition arising from the saturation of terrestrial ecosystems. According to Good *et al*. (2004), two approaches have been used to increase nutrient-use efficiency in crop plants: the use of traditional breeding and marker-assisted selection to identify genes involved in nutrient-use efficiency; and the use of gene constructs to improve specific aspects of nutrient use efficiency. In order to make significant progress in nutrient-use efficiency, they emphasized the need to combine genetic and transgenic approaches.

The definition of N-use efficiency varies with the type of study (reviewed in Good *et al*., 2004; Dawson *et al*., 2008). N-use efficiency is defined as the ability of a genotype to produce superior grain yields under low soil N conditions in comparison to other genotypes (Graham, 1984; Sattelmacher *et al*., 1994). It is a measure of the extent to which a crop plant transforms available N to economic yield (Ma and Dwyer, 1998). Moll *et al*. (1982) defined N-use efficiency as grain production per unit of N available in the soil. N-use efficiency can be divided into two processes: uptake efficiency which is the ability of the plant to remove N (normally present as nitrate or ammonium ions) from the soil; and utilization efficiency which is the ability of the plant to transfer the N (predominantly present as protein) to the grain (Moll *et al*.,

1982; Lea and Azevedo, 2006). N-uptake efficiency refers to the quantity of N absorbed by the plant relative to available soil N. At conditions of high N supply, uptake is mainly dependent on growth related demand for N, whereas at low N conditions, uptake is dependent on morphological and physiological root characteristics (Engels and Marschner, 1995). N-utilization efficiency quantifies the amount of grain produced per unit N uptake. It is influenced by the transportation, partitioning, and remobilization of N within the plant or cell, as well as specific metabolic processes at the cellular level (Engels and Marschner, 1995; Masclaux *et al*., 2001).

According to Moll *et al*. (1982), efficiency in uptake and utilization of N in the production of grain requires that those processes associated with absorption, translocation, assimilation, and redistribution of N operated effectively. In their study, they indicated that differences in N-use efficiency among six hybrids at low N levels were primarily due to variation in efficiency of utilization of N accumulated prior to anthesis, whereas hybrid differences at high levels of N were largely due to variation in N-uptake efficiency. Hirel *et al.* (2001) reported results which suggested that increased productivity in maize genotypes was due to their ability to accumulate nitrate in their leaves during vegetative growth and to effectively remobilize this stored N during grain filling. According to Parr (1973), factors which affect N-use efficiency include: source of N, rate of applied N, method and time of application, nature of soil, climatic conditions, genetic make-up and N requirement of the crop, extent of microbiological and chemical immobilization, and agronomic management factors such as weed control, seedbed preparation, timeliness in planting and harvesting.

According to Moll *et al*. (1982), N-use efficiency (NUE) is a product of N-uptake efficiency (NupE) and N-utilization efficiency (NutE). For grains:

$$\text{NupE} = \frac{\text{Nt}}{\text{Ns}} \text{ (g plant N /g supplied N)}$$

$$\text{NutE} = \frac{\text{GY}}{\text{Nt}} \text{ (g grain /g plant N)}$$

$$\text{NUE} = \text{NupE} \times \text{NutE or } \frac{\text{GY}}{\text{Ns}} \text{ (kg grain /kg supplied N)}$$

Where: Nt = total plant N; Ns = N supplied; GY = grain yield

However, following the procedure of Craswell and Goodwin (1984), the definitions of N-use efficiency and its components differ from that of Moll *et al*.

(1982) in that the analysis uses an unfertilized control as the initial starting point for analysis. Therefore,

$$\text{NupE} = \frac{\text{NCF} - \text{NCC}}{\text{NRF}} \text{ (g plant N /g supplied N)} \quad (1)$$

$$\text{NutE} = \frac{\text{GYLDF} - \text{GYLDC}}{\text{NCF} - \text{NCC}} \text{ (g grain /g plant N)} \quad (2)$$

$$\text{NUE} = \frac{\text{GYLDF} - \text{GYLDC}}{\text{NRF}} \text{ (kg grain /kg supplied N)} \quad (3)$$

Where: NCF = total N in plant for fertilized plot; NCC = total N in plant for control plot (no fertilizer): NRF = N fertilizer rate applied; GYLDF = grain yield of fertilized plot; GYLDC = grain yield in control plot (no fertilizer).

The appropriate way to estimate nutrient-use efficiency depends on the crop, its harvest product and whether the researcher is interested in analysing specific physiological processes involved in nutrient-use efficiency (Good *et al*., 2004).

2.5 Strategies for the improvement of N-use efficiency

Several strategies have been suggested for improving crop N-use efficiency. Regardless of the strategy adopted, the challenges to improving N-use efficiency include the optimization of N supply and demand, maximizing crop N-uptake and assimilation, minimizing N losses and ultimately, specific improvements in the yields of biomass, leaves, fruits or seed (Pathak *et al*., 2008).

The options available for the improvement of N-use efficiency include: reducing the application of N (Fernandez *et al*., 1998); optimizing the timing of N application (Raun and Johnson, 1999); use of organic manure (Kumar and Goh, 2000; Nicholson *et al*., 2003; Papastylianou, 2004); symbiotic nitrogen fixation (James and Olivares, 1998); crop rotation using legumes (Pikul *et al*., 2005); genetic selection (Lafitte and Edmeades, 1994a; Machado and Fernandez, 2001; Kamara *et al*., 2003, 2005); plant transformation by the insertion of new genes (Andrews *et al*., 2004) as well as increasing the genetic capacity of the maize plants to capture and utilize soil and fertilizer N before it is lost from the soil profile (Oikeh *et al*., 2003). However, the use of inorganic fertilizers in Africa is limited because of high costs and inefficient marketing systems (Honlonkou *et al*., 1999; Lafitte and Edmeades, 1994a). The high cost makes N fertilizer too expensive for the resource-poor farmers who due to the latter apply nitrogen at sub-optimal levels (McCown *et al*., 1992). Organic manure is

usually required in large quantities to sustain crop production; the quantity required is usually not readily available to small-scale farmers (Nyathi and Campbell, 1995). This is compounded by the demanding nature of compost preparation, which makes it unappealing to the farmers. The use of a mixture of organic and inorganic fertilizer (organomineral fertilizer) has been suggested as best for the soils of the humid tropics (Agboola and Odeyemi, 1972; Agboola, 1982; Palm *et al*., 1997; FAO, 1999), where it has been shown to increase the nutrient-use efficiency of plants (Murwira and Kirchmann, 1993) as well as crop yield, soil fertility status or both (Palm *et al*., 1997), However, organomineral fertilizer use is still limited. Symbiotic nitrogen fixation has been estimated to contribute about half of the amount of N applied in inorganic N fertilizers (Smil, 2006) and may be an ecological alternative to inorganic N fertilizer application in several areas of the world (Shantharam and Mattoo, 1997). The use of symbiotic N fixation alone in large scale production systems is neither practical nor sustainable in meeting the production needs in the savannas of Africa. The possibility of using nitrogen-fixing legumes in rotation is not sustainable as the amount of soil nutrient supplied in those systems is not enough to solve the production problems in the African savannas because of exports through harvests (Carsky and Iwuafor, 1999).

Though the form and amount of N available to the plant can be managed by managing fertilizer-soil-water-air interactions, the innate efficiency of the plant to utilize available N have to be tackled biologically (Pathak *et al*., 2008). Thus, there is the need to develop new cultivars more efficient in the conversion of fertilizer N into harvestable yield (Rizzi *et al*., 1993).

2.6 The biology of nitrogen use efficiency

Several studies have been carried out which reported the performance/response of different maize genotypes to varying levels of N application and environments (Akintoye *et al*., 1999; Bänziger *et al*., 1997, 1999; Bertin and Gallais, 2000; Coque and Gallais, 2007b; Kamara *et al*., 2005; Worku *et al*., 2007). These studies and a wide range of others, which have been extensively reviewed by Gallais and Coque (2005), have shown the existence of genetic variation for N responsive traits in maize.

NUE as a quantitative trait is controlled by the actions and interactions of several genes involved in the processes responsible for the acquisition of N from the soil, assimilation into amino acids, and transport and use of N (Moose and Below, 2008). The characterization of the biological responses of maize to N under controlled

environment is very crucial to measuring the genetic aspect of N-use efficiency. Good *et al*. (2004) and Pathak *et al*. (2008) reviewed recent advances in molecular biology, genetics and functional genomics that could or have been applied to better understand maize N metabolism and identify genes whose expression could be modified to improve N-use efficiency. While in the soil, nitrate is the preferred N source for plants. Nitrate is taken up by active transport into the roots, assimilated by the actions of nitrate reductase, which reduces nitrate into nitrite, and nitrite reductase, which reduces nitrite into ammonium (Meyer and Stitt, 2001). The ammonium produced is incorporated into amino acids via the glutamine synthetase and glutamate synthase (GOGAT) cycle (Lea and Miflin, 2003). An illustration of the important processes, traits, pathways, and classes of genes whose expression have either been demonstrated or expected to contribute to maize N-use efficiency is presented in Figure 2.1.

2.6.1 N uptake and assimilation

Nitrate is taken up by plant roots by the combined action of a set of both high- and low affinity transporter systems (Chopin *et al*., 2007). The high affinity transporters (HATS), which are induced, are preferentially used under low external nitrate concentrations while the low affinity transporters (LATS), which are constitutive, are used when external N concentration is high (Glass and Siddiqi, 1995). Rapid assimilation of the absorbed N into amino acids occurs and in maize, this takes place primarily in the leaves. The nitrate is rapidly reduced to ammonia and is then combined with glutamate to form glutamine. The bulk of the amino acids found in the source tissues are glutamine, glutamate, alanine, aspartate and the storage amino acid asparagine. The key function of these metabolites, which are constituents of the asparagine cycling pathway, in regulating carbon-nitrogen balance among higher plants has been reported (Galili *et al*., 2008). Thus, as schematically represented in Figure 2.2, the key enzymes involved in primary N assimilation and interconversion among the major amino acid carriers are nitrate reductase, glutamine synthetase, alanine aminotransferase, aspartate aminotransferase, and aspargine synthetase (Moose and Below, 2008).

2.6.2 N transport

N uptake and recycling proceed through four developmental phases namely early seedling growth phase, pre-flowering vegetative N accumulation phase, post flowering N accumulation phase and grain filling N remobilization phase. Seedling

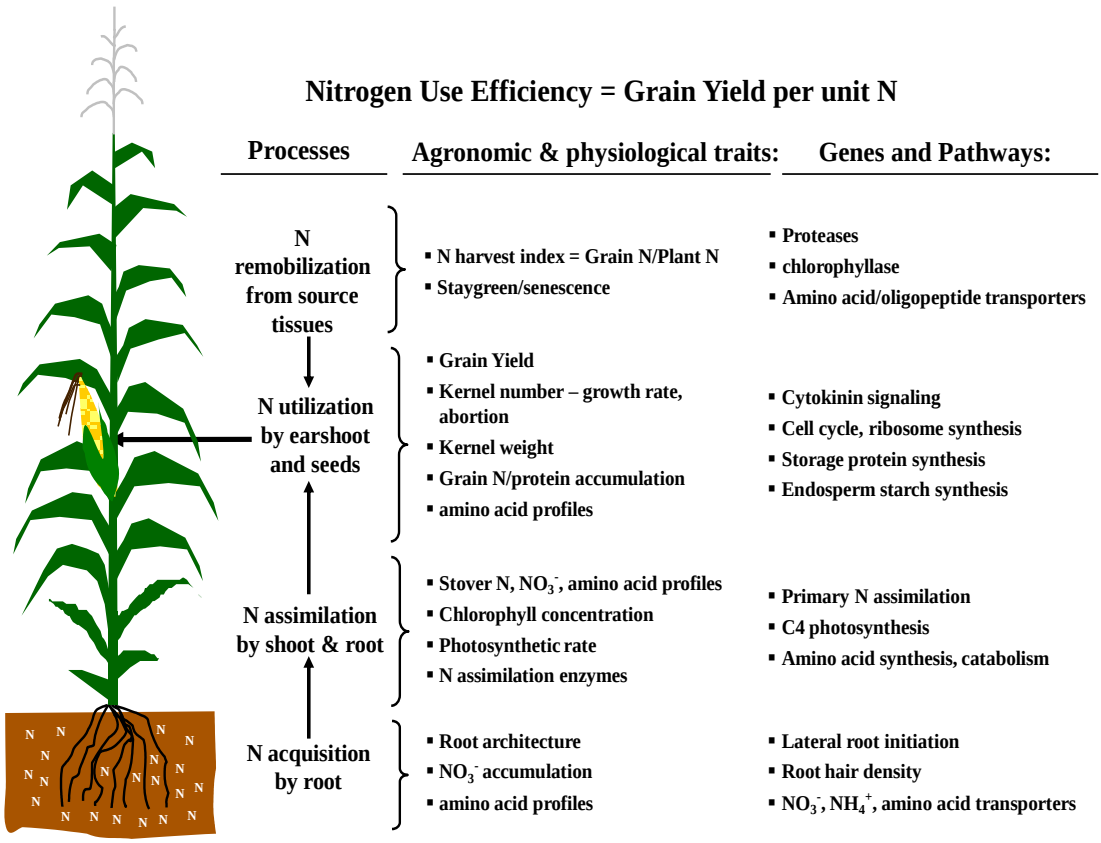

Figure 2.1. Major physiological processes, observed phenotypes, and genetic pathways associated with maize nitrogen-use efficiency (adapted from Moose and Below, 2008).

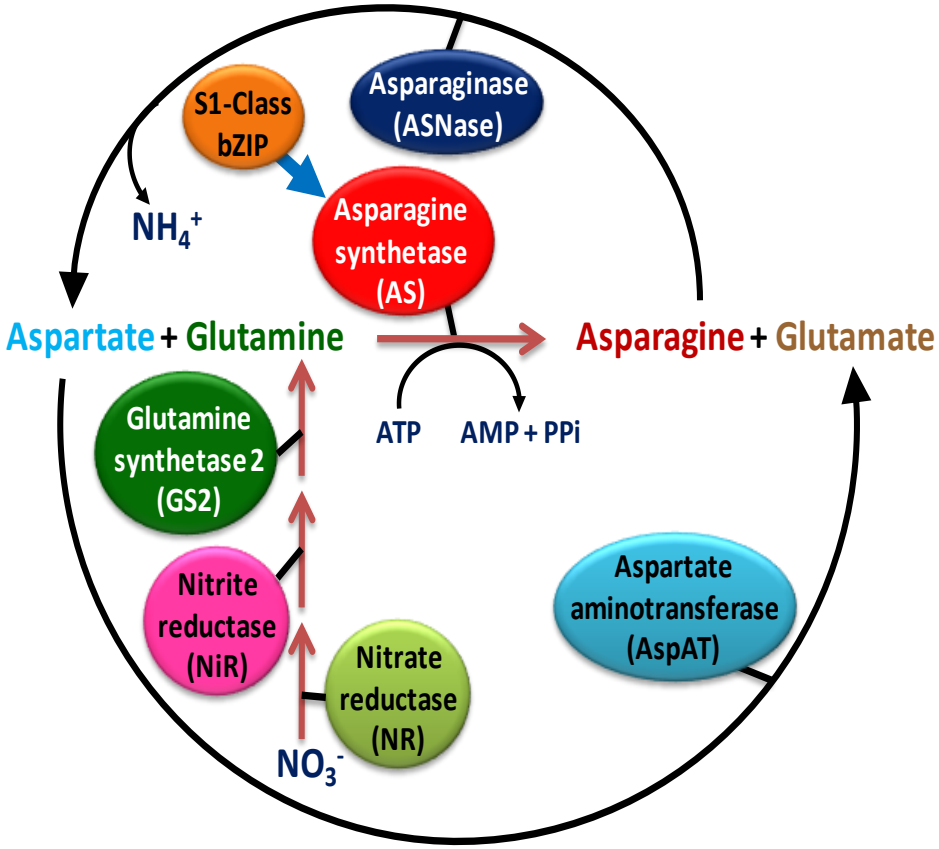

Figure 2.2. The asparagine cycling pathway. Gene products are shown as filled coloured circles, metabolites as coloured text. ATP=Adenosine Triphosphate; AMP=Adenosine Monophosphate; PPi=Pyrophophate.

growth in the early stages is supported by the release of N from the breakdown of seed N reserve which must be accounted for in studies of N metabolism (Moose and Below, 2008). During the pre-flowering vegetative growth, substantial amount of N is accumulated and at silking, depending on genotype and environmental conditions, about 50 – 75% of the total plant N accumulated at maturity is already present (Gallais and Coque, 2005). N-uptake from the soil continues for a brief period after flowering and then declines with newly acquired N being directed to the developing seeds (Bertin and Gallais, 2000; Gallais and Coque, 2005; Moose and Below, 2008). More than 50% of amino acids synthesized from post-flowering N-uptake are first integrated into the stover proteins before translocation to the grains (Gallais *et al*., 2006). Although genotypic and environmental variations do exist for the proportion, the bulk of N that supports kernel development is remobilized from the stover. Studies (Ta and Weiland, 1992; Rajcan and Tollenaar, 1999b; Gallais and Coque, 2005) have shown that 45 – 65% of grain N is provided from pre-existing N in the stover at silking while the remaining 35 – 55% originates from post-silking N-uptake. Traits that have been associated with continued N accumulation during grain filling include increased grain protein concentration and delayed leaf senescence (Moose and Below, 2008). N remobilization involves the transport of free amino acids, amino acids from the breakdown of proteins and recycled chlorophyll to developing kernels. It is noteworthy that the transfer of N to developing kernels is almost without dry matter transfer and not all stover N is remobilized at maturity (Gallais and Coque, 2005). The entire process of N remobilization involves the activities of proteases, glutamine synthetase (GS), glutamate dehydrogenase (GDH), amino acid transporters, and the chlorophyll degradation pathway (Hirel *et al*., 2005; Moose and Below, 2008).

2.6.3 Regulation of N–associated processes

The regulation of nitrate assimilation is influenced by both endogenous and exogenous factors including nitrate, CO_2, light, hormones, temperature, carbon and nitrogen metabolites (Sivasankar and Oaks, 1996; Moose and Below, 2008). Although nitrate serves as a major source of N for plants, it is used by plants as a signal to reprogram N metabolism (Forde, 2002; Glass *et al*., 2001). Both the external (Forde, 2000) and internal (Forde and Clarkson, 1999) pools of nitrate are involved in the feedback regulation of N metabolism. Amino acids play important role in N metabolism and signaling. Studies (Seebauer *et al*., 2004) have shown that the ratio of

glutamine to asparagine can potentially serve as an indication of plant N status. Similarly, the role of glutamate as a signaling molecule in N metabolism was reviewed by Forde and Lea (2007). N metabolism is also dependent on signaling action by light (Lillo and Appenroth, 2001; Raghuram *et al*., 1999), cytokinins and polyamines (Inoue *et al*., 2001; Sakakibara *et al*., 2006). Studies have also shown the involvement of some N- and/or C- responsive regulatory proteins such as G-protein (Ali *et al*., 2007; Raghuram *et al*., 1999), 14-3-3 proteins (Comparot *et al*., 2003), and PII proteins (Hsieh *et al*., 1998), among others, in the regulation of N metabolism.

2.7 The field of plant genomics

The study of genes way genes and genetic information are organized within the genome, the methods of collecting and analyzing this information and how this organization determines their biological functionality is referred to as genomics. Plant genomics focuses on finding biological functions behind genes. It bridges the gap between phenotype and genotype and helps to comprehend not only the isolated effect of a gene, but also the way its genetic context and the genetic networks it interacts with can modulate its activity (Campos-de Quiroz, 2002).

Genomics combines three areas that focus on the science and technology of organization of the genome (Tinker, 2002; Johnson, 2004). The first component is genetics, which is the study of the particulate nature of inheritance. The second component is automated laboratory tools for high throughput DNA-, RNA-, and protein analyses. These depend on the growing knowledge of nucleic acid biochemistry. The third component is bioinformatics, which is the application of information science to sequence level genetic information and molecular genetics. It resides at the intersection of biology, mathematics, statistics, and computer science, and uses information science to identify and align sequences, to predict how genes or their products interact to create genetic networks. Bioinformatics provides the tools that enable the molecular description of the genetic bases of phenotypes, and facilitates predicting phenotypes from gene sequences and associated information.

Genomics can be divided into structural and functional components (Edmeades *et al*., 2004). Structural genomics deals with the physical structure of the genome, including the sequence and organization of genes. The generation of physical maps of gene locations on chromosomes, and information on the structure of genes themselves, including promoter regions, coding regions and terminator sequences are examples of

products arising from structural genomics. Functional genomics focuses on gene products, transcripts and gene interactions. Linkage maps, gene × gene interaction information, gene expression profiles and the association of specific genes with a corresponding phenotype are typical products of functional genomics. Gene-to-phenotype associations, such as quantitative trait loci (QTLs), can themselves suggest candidate genes. These in turn can be located on the physical map, modified, placed under transcriptional control of different promoter sequences, and inserted back into a host genome to determine phenotypic effects of over- or under-expression of the gene. Thus, functional and structural genomics complement each other in the search for the basis of gene-to-phenotype relationships

2.8 The area of functional genomics

The term functional genomics, according to Hieter and Boguski (1997), refers to the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics. It is an area of genome research that is concerned with assigning biological functions to DNA sequences (Yang and Speed, 2002). Functional genomics employs multiple parallel approaches and tools such as expression sequence tag (EST) generation, global transcript profiling, transgenics and reverse and forward genetics, for high throughput studies of gene function (Coram *et al*., 2007; Vij and Tyagi, 2007). Gene function can be considered from several points of view: it may mean biochemical function (e.g. protein kinase), cellular function (e.g. a role in signal transduction pathway), developmental function (e.g. a role in pattern formation), or adaptive function (the contribution of the gene product to the fitness of the organism) (Bouchez and Höfte, 1998). The aim of functional genomics is to link the genome to the phenome (phenotype).

Functional genomics can broadly be divided into three different areas, namely, transcriptomics, metabolomics, and proteomics. Transcriptomics or transcriptional profiling deals with the generation and analyses of gene expression patterns across a wide array of cellular responses, phenotypes and conditions. Similarly, metabolomics and proteomics involve the global expression profiling of specific metabolites or proteins respectively, from a specific tissue/organ in response to a treatment.

There are several systems available to analyse the parallel expression of many genes, and these includes macroarrays (Desprez *et al*., 1998), microarrays (Schena *et*

al., 1995), Serial Analyses of Gene Expression (SAGE) (Velculescu *et al*., 1995), Massively Parallel Signature Sequencing (MPSS) (Brenner *et al*., 2000a), GeneCalling technology (Bruce *et al*., 2000), two dimensional gel electrophoresis (2DGE) (Rabilloud, 2002; Lilley *et al*., 2002) and Yeast two-hybrid expression (Chern *et al*., 2007; Miller and Stagljar, 2004). Functional genomics has been widely used for studying the stress responses of plants such as tomato (Gibly *et al*., 2004), rice (Fujiwara *et al*., 2004), maize (Baldwin, 1998), cassava (Lopez *et al*., 2005), soybean (Moy *et al*., 2004), and *Arabidopsis thaliana* (Huitema *et al*., 2003), to mention but a few.

2.9 Technologies for measuring gene expression

Several technologies or approaches have been employed to study gene expression. These include RNA-based (Hauser *et al*., 1997), sequence-based (Velculescu *et al*., 1995; Brenner *et al*., 2000b), fragment-based (Bachem *et al*., 1996; Shimkets *et al*., 1999), and hybridization-based such as macro- and microarrays (Schena *et al*., 1995; Lockhart *et al*., 1996; Desprez *et al*., 1998).

Differential gene expression profiling technologies can be divided into two major groups namely 'closed architecture' systems and 'open architecture' systems. Closed architecture systems requires *a priori* sequence knowledge of each gene or clone to be assayed and only measures differences in gene expression in a limited set of selected or known genes e.g. microarrays and quantitative RT-PCR strategies. Open architecture systems (e.g. differential display) do not assume prior sequence knowledge and can, theoretically, survey all transcripts in any selected tissue and identify novel genes.

RNA gel blot analysis is a reliable and sensitive method for quantifying mRNA abundance in plant tissues and allows for the accurate quantification of specific transcripts (Hauser *et al*., 1997). The RNA gel blot is a technique in which a labeled probe is hybridized to a RNA target, and the resulting band size and signal intensity is used to confirm and quantify expression. This method is however scale limited and therefore cannot be readily adapted to genome-scale analysis (Alba *et al*., 2004).

Differential display (Liang and Pardee, 1992; Welsh *et al*., 1992) is an expression analysis method whereby mRNA from each sample is converted to cDNA. The cDNA is PCR amplified using a combination of random primers and anchored oligo-dT primers, then separated by gel electrophoresis. Each mRNA is represented on

a single band and differentially expressed bands are excised, cloned, and sequenced to reveal identity. The technique requires minimal mRNA and allows for parallel profiling of mRNA populations. However, the output from this method is not quantitative and positives are difficult to clone and confirm (Ding and Cantor, 2004). This technique can easily be performed in any laboratory equipped with standard molecular biology reagents and instrumentation.

cDNA – AFLP transcript imaging (Bachem *et al*., 1998) is a technique used for the visualization of differential gene expression, It involves the application of the principles of AFLP to cDNA templates. This technique allows for the investigation of poorly characterized genomes in a high throughput manner: a wide variety of tissue types, developmental stages or time point to be concurrently compared. The cDNA – AFLP technique has been used to investigate differentially expressed genes involved in a variety of plant processes (Bachem *et al*., 2001; Dellaqi *et al*., 2000; Durrant *et al*., 2000; Qin *et al*., 2000).

GeneCalling (Shimkets *et al*., 1999) is a quantitative differential gene expression analysis technique adapted for high sample throughput. The technique subjects cDNA samples to different enzymes simultaneously. Fragments are amplified using fluorescently-tagged primers and these PCR fragments are separated by capillary electrophoresis and the precise size of each fragment is concurrently determined. Trace data are generated for each paired digest reaction and fragments are visualized. By electronically comparing the relative intensity of each peak between a control and an experimental sample, cDNA fragments derived from differentially expressed genes are automatically identified. As GeneCalling exploits but does not require advance sequence information, the technique can be complemented by the generation of custom data bases (Green *et al*., 2001).

cDNA sequencing provides a more direct and comprehensive approach to gene expression profiling (Adams *et al*., 1991; Okubo *et al*., 1992). The technique however requires substantial resources for cloning and sequencing and is less sensitive to low-abundance transcripts.

Serial Analysis of Gene Expression (SAGE) is a tag-based method that provides a quantitative estimate of gene expression (Velculescu *et al*., 1995). It combines differential display and cDNA sequencing approaches. The technique depends on the generation of unique transcript-specific short sequence tags of 9 – 17 base pairs (Saha *et al*., 2002). The quantification of a particular tag provides the

expression level of the corresponding transcript. SAGE has been used to study global gene expression profiling of rice (Matsumura *et al*., 1999) and gene expression in response to cold stress in *Arabidopsis thaliana* (Jung *et al*., 2003; Lee and Lee, 2003). The SAGE method is however laborious, requires extensive foundation of sequence information and its sensitivity to low-abundance transcript is a serious concern.

SuperSAGE is a variant of SAGE that uses 26 base pair tags and is developed to address the apparent lack of specificity achieved with 9 – 17 base pair tags (Matsumura *et al*., 2003, 2008). The technique allows various applications which include interaction transcriptome and SuperSAGE array. According to Matsumura *et al*. (2008), SuperSAGE is perfectly complemented by emerging "Next Generation Sequencing" technologies, a combination that has generated a novel transcriptome platform superior to other microarray variants in terms of throughput, data quality and cost analysis. This revised technique has been used to investigate salt, drought and cold stress response in chicken pea (Kahl *et al*., 2007).

Massively Parallel Signature Sequencing (MPSS) is also a tag-based method and one of the most powerful technologies for the quantitative analysis of gene expression (Brenner *et al*., 2000a, b). It involves the cloning of a cDNA library on beads and the acquisition of 17 – 20nt 'signatures' (tags) from the cDNAs using an unconventional sequencing method. The abundance of the sequence signatures precisely reflects gene expression levels in the sampled tissue. This approach has well been used in the analysis of gene expression in *Arabidopsis thaliana* (Meyers *et al*., 2004). Due to the high cost of MPSS, the approach is not commonly used for transcriptional profiling.

Microarrays are designed for the simultaneous measurement of the expression of several thousands of genes in a single hybridization procedure (Schena *et al*., 1995). Probes derived from gene sequences or expressed sequence tags (ESTs) are immobilized on a solid surface and used to generate expression profile of a target sample *via* hybridization (Chen *et al*., 1998). Fluorescently labeled DNA or RNA derived from mRNA is then hybridized to their complementary DNA on the microarray and detected via laser scanning. Differences in labeling intensity are converted into quantitative output of relative gene expression. The advantages of microarrays lie in its speed and sensitivity. Reported detection levels are 1:300,000 for low abundance mRNAs (Lockhart *et al*., 1996).

2.10 DNA Microarrays

Microarray experiments are used to quantify and compare gene expression on a large scale. It allows the simultaneous monitoring of the expression levels of numerous genes (Schena *et al*., 1995; Yang and Speed, 2002). Microarrays offer the promise of high throughput parallel assessment of gene expression for large number of genes or cDNA fragments in different tissues and organisms (Schena *et al*., 1995). Actual gene expression is measured at a particular time, under appropriate conditions and related to traits of interest, often by comparing two groups differing for the trait (Chen *et al*., 2002; Gracey and Cossins, 2003).

Microarrays have revolutionized global gene expression profiling making it possible to study all genes of an organism in parallel if the entire genome is already sequenced (Wang *et al*., 2003). Microarrays have been used extensively for global expression profiling of plant responses to biotic and abiotic stresses. They use hundreds of highly organized probes printed on a solid surface to simultaneously interrogate the multiple RNA or DNA molecules defined as targets, within each sample (Schena *et al*., 1995). The target molecules are fluorescently labeled and hybridized to the immobilized probes. The signal generated from each probe-target hybrid is quantified and the strength of the signal represents (i) target abundance (transcript level, if transcripts were RNAs) or (ii) sequence similarity between probes and targets (Clarke and Zhu, 2006).

The potential of DNA microarrays to monitor the expression of thousands of targets simultaneously in a high-throughput manner facilitates recognition of global gene expression patterns. The comparison of expression patterns across different samples allows for the association of specific traits with changes in gene expression, suggesting gene function (Chen *et al*., 2002), as well as possible leads for plant improvement (Gutterson and Zhang, 2004). On global scale, DNA microarray technology has the potential to reveal the actual state of the transcriptome (Schmid *et al*., 2005), allowing for a better understanding of gene regulation at the system level.

2.10.1 Types of microarrays

Three types of microarrays can be identified based on the type of probe, namely: cDNA (spotted) microarrays, oligonucleotide (Gene Chip) microarrays, and tilling-path microarrays.

cDNA microarrays can be prepared directly from existing cDNA libraries.

They use cDNAs generated from mRNAs as probes. cDNA microarray experiments typically involve hybridizing two mRNA samples, each of which have been converted into cDNA and labeled with its own fluorophore, on a single glass slide that has been spotted with 10,000 – 20,000 cDNA probes (Yang and Speed, 2002). The fabrication of cDNA microarrays depends on the availability of ordered clone collections and appropriate arraying and scanning instrumentation (Clark *et al*., 1999; Eisen and Brown, 1999). The system has high sensitivity and data obtained highly reproducible. The system however suffers from high cost, the need for specialized arraying and scanning instrumentation, and the fact that the arrays are not re-useable (Bouchez and Höfte, 1998).

Oligonucleotide microarrays use short oligos (~60bp) designed from known gene/DNA sequence as probes. High density oligonucleotide microarray experiments provide direct information about the expression levels in an mRNA sample of the 200,000 – 500,000 probe gene fragments (Redfern *et al*., 2000). Fabrication of oligonucleotide arrays depends on the availability of required gene sequences and appropriate arraying and scanning instrumentation. The short oligos can be synthesized directly on glass under controlled conditions (McGall *et al*., 1996). Alternatively, oligonucleotide arrays may be fabricated photolithographically, which is a microfluidic technology that utilizes light to direct the synthesis of short oligos into a suitable matrix (Pease *et al*.,, 1994). The use of short oligo probes requires that a minimum of 9 to 11 independent probes per gene sequence is necessary to accurately measure the transcript abundance without a significant deterioration in performance (Zhou and Abagyan, 2002). Since oligo arrays are designed to represent unique gene sequences, cross hybridization between related gene sequences is minimized. There is also increased statistical power and precision as a result of reduced experimental variation. The technology however, could be very expensive, and potentially vulnerable to single base changes due to polymorphism or sequence error in the original sequence used for oligo design (Alba *et al*., 2004, Mah *et al*., 2004).

Tiling-path arrays represent the complete genome, including intergenic regions, by probes on the array, rather than only using gene-specific probes to detect gene expression (Rensink and Buell, 2005). Tiling arrays, in addition to detecting transcripts, may be used for comparative genome hybridization to detect deletions and polymorphisms, methylation profiling, and analysis of chromatin immuno-precipitation samples (Martienssen *et al*., 2005). The use of tiling-path arrays is

limited to availability of entire genome sequence, which is currently only possible in model plants such as *Arabidopsis* and rice. Tiling arrays have the advantage of being less biased for the expressed sequence as no assumption is made about the gene structure and which part of the genome sequence is coding.

2.11 cDNA microarrays

2.11.1 Fabrication

The fabrication of cDNA microarray usually involves the generation of a cDNA library for the experimental purpose and the selection of clones to be queried. These clones can be sequenced from the 3' and/or 5' end and annotated by blasting the sequence to the GenBank® databases. Clones with known function, also referred to as ESTs, are then spotted in a matrix on a solid platform (Duggan *et al*., 1999).

2.11.2 Experimental design

Alba *et al*. (2004) presented a schematic overview of experimental design for gene expression using cDNA microarrays (Figure 2.3). Various experimental designs are possible for microarray analysis, most of which have been discussed in detail (Churchill, 2002; Dobbin and Simon, 2002; Yang and Speed, 2002; Dobbin *et al*., 2003; Clarke and Zhu, 2006). The experimental designs well suited for gene expression profiling during time-course studies or analysis of developmental transitions (Alba *et al.*, 2004) are the direct-sequential linear design and the direct-sequential loop design (Kerr and Churchill, 2001; Yang and Speed, 2002). More recently, experimental designs for microarray analyses have begun to incorporate interspecies comparisons using arrays that originate from close relatives of the genomes being investigated (Dong *et al*., 2001; Horvath *et al*., 2003; Ventelon-Debout *et al*., 2003). The comparison of closely related species is most effective and informative because artifacts stemming from sequence divergence are minimized. An example of this type of comparison is co-hybridization of cDNA derived from pepper and tomato pericarp onto a tomato TOMI microarray to study gene expression (Alba *et al*., 2004).

2.11.3 Generation of hypothesis

A well-designed expression profile experiment built around a hypothesis yields high quality results that lend themselves to validation. Microarray experiments can be categorized as hypothesis seeking and hypothesis testing (Clarke and Zhu, 2006).

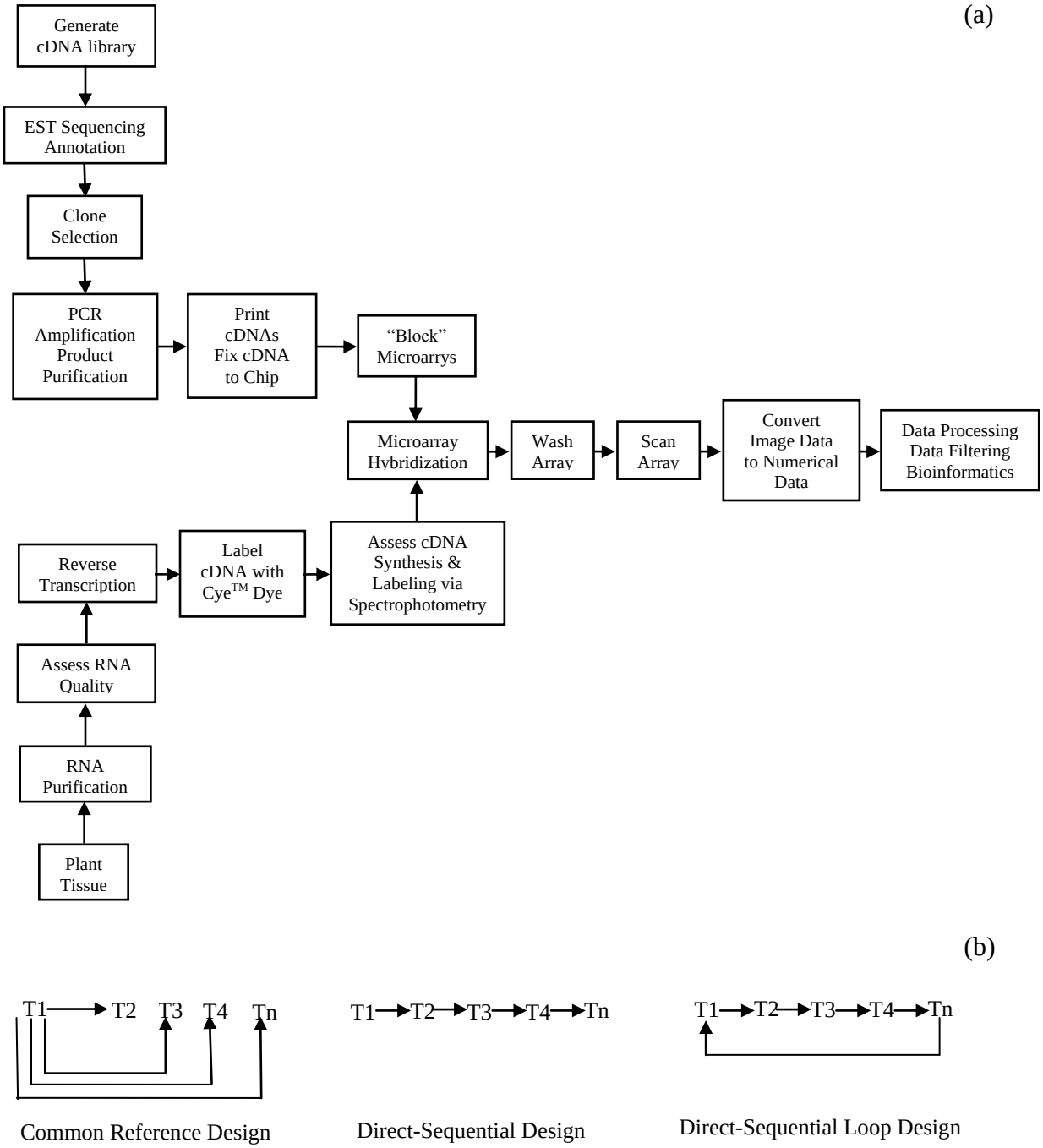

Figure 2.3. Overview of experimental design for gene expression profiling using cDNA microarrays. (a) General scheme for gene expression profiling using cDNA microarrays. (b) Three different experimental designs for time-course experiments utilising microarrays. Abbreviations: T1……Tn, time-points 1 through n (adapted from: Alba *et al*., 2004).

Hypothesis seeking begins with minimal information about the subject, followed by the gathering of information through transcriptional profiling and a working hypothesis built to validate particular gene function. Hypothesis testing on the other hand begins with a specific question and interrogates the transcriptional changes between conditions to address that question.

2.11.4 Sources of variation

Microarray experiments are a multi-step process and each step is a potential source of variation. Variability can be generally classified into three categories: technical variation, biological variation and residual variance (Novak *et al*., 2002; Churchill, 2002). Technical variation refers to the variation arising from the use of the microarray system. Potential sources of technical variation include the: sample preparation procedures, microarray construction procedures, hybridization and washing procedures, detection method, as well as laboratory environmental conditions. Biological variation refers to the variation from different RNA sources. It reflects differences in host characteristics. Biological variation is due to inherent differences in gene expression, varying from subject to subject due to genetic or environmental factors (Chen *et al*., 2004). Biological variation can be divided into intra- and inter-sample variation. Intra-sample variation could arise as a result of micro-environmental differences within the same sample, while inter-sample variation could arise due to environmental differences caused by growth room/greenhouse or field effects. Sample pooling and replication are the primary methods used to account for biological variation (Clarke and Zhu, 2006). In an analysis using high- quality microarrays, biological variation far exceeds technical variation and remains the main concern surrounding microarray experiments (Bakay *et al*., 2002).

2.11.5 Replication

Replication allows for the assessment of variability of expression data such that formal statistical analysis methods can be applied. Without replications, it is difficult to distinguish between the true differences in gene expression and random fluctuations (Chen *et al*., 2004). Yang and Speed (2002) described two types of replications namely: technical and biological replications. Technical replication refers to replication in which the mRNA is from the same extraction, while biological replication refers to hybridizations that involves mRNA from different extractions. Technical variation is minimal for in-house synthesized oligo- and cDNA-arrays, thus,

in designing microarray experiments, biological replication is a priority (Zhu and Wang, 2000).

In any meaningful transcriptome profiling, sufficient replication is an important issue and decisions in this regard should be based on the (i) extent of biological and technical variation (ii) experimental question (iii) desired resolution (iv) available resources (v) available time and (vi) opportunities for downstream validation (Alba *et al*., 2004). Currently, a minimum of three or four biological replications with a dye-swap per time point is recommended to accommodate variation (Lee *et al*., 2000; Kerr *et al*., 2002). Dye-swap is helpful in reducing dye bias that is derived from differences in the mean brightness and background noise of individual spots, incorporation efficiencies, extinction coefficients, quantum fluorescence yield and other physical properties of the dyes (Tseng *et al*., 2001; Yang *et al*., 2001). It should be noted that, while replication can estimate but not remove variation in experimentation; therefore, efforts should be directed at creating a uniform environment for samples to facilitate downstream data analysis (Clarke and Zhu, 2006).

2.11.6 RNA extraction, target preparation and hybridization

cDNA microarrays are commonly performed in a reference design where mRNAs from test and reference samples are hybridized onto the same probe-set. This allows comparison of the relative transcript abundance of the test sample to the reference sample. Reference and test samples should be collected from plants grown under the same conditions but differ only in the treatment being investigated, to have an effective comparison. Extraction of RNA from collected tissue samples is carried out using phenol-based extraction methods, guanidine thiocyanate, TRizol®, silica-based extraction (e.g. RNeasy columns; Qiagen, Inc., Valencia, CA, USA), and methods that use proprietary extraction cocktails such as RNAwiz (Ambion, Austin, TX, USA). The purity and integrity of RNA can influence cDNA synthesis, incorporation of fluorescent dyes, dye stability and probe-target hybridization. Therefore, extensive purification of RNA is essential to remove all contaminating protein, polysaccharides and other organic materials especially RNases (Holloway *et al*., 2002). RNA purity and integrity is generally verified using a spectrophotometer and formaldehyde denaturing gel electrophoresis before reverse transcription to target cDNAs (Alba et al., 2004).

Labeling of test and reference targets is carried out using fluorescent dyes, usually cyanamine-3 (Cy3) and cyanamine-5 (Cy5) (Duggan *et al*., 1999) using the direct or highly preferred indirect (amino allyl) labeling technique (Holloway *et al*., 2002). Dye incorporation is assessed by spectrophotometry and equal amounts of test and reference targets is hybridized onto the spotted array under cover slip in a special chamber (e.g. Corning®, hybridization chamber) to avoid evaporation (Coram and Pang, 2006). The hybridization temperature ranges from 42^{o}C (if using 50% formamide) to 70^{o}C (if using SSC-based buffers) and incubation duration varies from several hours to overnight (Aharoni and Vorst, 2001).

2.11.7 Acquisition, transformation and normalization of data

There exist a wide range of protocols and equipment for acquisition and downstream processing of microarray data. Alba *et al*. (2004) presented a list of bioinformatics tools and resources for microarrays. Slides are scanned immediately after they are washed of excess hybridization solutions and dyes, and dried, using a two-channel confocal microarray scanner (e.g. Affymetrix 428^{TM} array scanner, Santa Clara, CA; Genepix 4000B scanner, Molecular Devices Corporation, Sunnyvale, CA; ScanArray 5000, GSI Lumonics, Billerica, MA, USA) and associated software. Subsequent to laser focusing and balancing, scans are conducted at 10 µm resolution with the laser power typically set between 70 and 85% of maximum and the photomultiplier tube set at 80% of maximum. Excitation settings used for cyanine-3 (Cy3^{TM}) and cyanine-5 (Cy5^{TM}) fluors are 543/570 µm and 633/670 µm respectively, and both fluorescent image files saved as .tiff files. The raw image data is digitally quantified using another software (e.g. Genepix Pro software, Molecular Devices Corporation, Sunnyvale, CA; ImageneTM software, BioDiscovery Inc., El Segundo, CA, USA) and the gene expression values saved as .txt
files and/or .xml files.

The processing of microarray data usually involves correction for background signals, omission of flagged spots, transformation and normalization. Data transformation and normalization allows for the detection of actual (biological) variation in gene expression and its distinction from experimental and systematic variation. Quackenbush (2002) reviewed the various microarray data transformation and normalization techniques available. In comparing five commonly used

transformation methods, Alba *et al*. (2004) found log transformation to be the most reliable. According to Draghici (2003), $\log_2$ transformation is most commonly used because it converts the gene expression values to an intuitive linear scale representing two-fold differences.

Normalization of gene expression data is carried out to remove or minimize and standardize systematic (non biological) variation that exists in experimental data sets (Cui and Churchill, 2003; Chou *et al*., 2005; Clarke and Zhu, 2006) and it accounts for factors such as background intensity, noise levels, differences in measurements, hybridization conditions and variations caused by handling (Leung and Cavalieri, 2003). The appropriate use of normalization permits comparisons between arrays within an experiment and possibly between arrays from separate experiments (Clarke and Zhu, 2006). Comparing different normalization techniques, Alba *et al*. (2004) showed that the LOcally WEighted polynomial regreSSion (LOWESS) was most suitable. This method of normalization uses a locally weighted regression that reduces the expression ratios to the residual of the lowest fit of an associated intensity versus ratio curve (Cleveland and Devin, 1988).

2.11.8 Candidate gene selection and statistical analyses

Reports on microarray experiments have generally based selection of candidate genes on gene annotation, transcript level, or fold difference. Although the ′two-fold or more′ cut-off is widely preferred by molecular biologists, its use should be applied with some caution, as it could be misleading in the event of one of the candidate genes having transcript levels below the threshold or above saturation, leading to over- or under- estimation of fold change (Leung and Cavalieri, 2003; Clarke and Zhu, 2006). The proper use of biological replication which permits statistical analyses can greatly improve fold
change data (Lee *et al*., 2000).

Statistical analyses of microarray data employs either parametric and non-parametric methods to determine if the probabilities that observed differences in expression values are real or false positives as a consequence of random variation (Clarke and Zhu, 2006). The parametric methods most commonly employed are the Student's *t*-test (assumes equal variances) and Welch's *t*-test (assumes unequal variances) when comparing two groups, and analysis of variance (ANOVA) when more than two groups are to be compared. Non-parametric methods include Wilcoxon

rank sum test and the Kruskal-Wallis test when comparing two and more than two groups respectively. There abounds a lot of information in the literature on statistical tests for microarray experiments, as reviewed in Nadon and Shoemaker (2002); Cui and Churchill (2003), Draghici (2003), Leung and Cavalieri (2003), and Wu (2009) among others.

2.11.9 Validation of microarray data

The aim of microarray data validation is to ascertain that the differential expression detected by the array is repeatable by other means. Validation of microarray data can be experimentally achieved by methods such as RNA blot analysis, real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, and data mining. The relative expression levels detected by RNA-blot are quite similar to those of microarrays. On the other hand, real-time qRT-PCR is more accurate and sensitive than microarrays (Czechowski, *et al*., 2004; Li *et al*., 2010) and often yields expression levels that differ in magnitude but similar in expression patterns (Czechowski, *et al*., 2004; Dallas *et al*., 2005). Data mining could also be employed to ascertain the expression of a particular gene under a given condition by searching existing expression data in public data repositories such as NCBI, and the Gene Expression Omnibus. Coupled with gene annotation, these data could be used to aid the functional discovery of gene function in response to a condition (Rensink and Buell, 2005).

CHAPTER 3
MATERIALS AND METHODS

3.1 Location of experimental site

The research work was carried out at the experimental station of the International Institute of Tropical Agriculture (IITA) in Mokwa (9º 18′N, 5º 04′E, and 457masl) located in the southern Guinea savanna ecology of Nigeria. The Guinea savanna ecology has been identified as the most suitable for maize grain production in Nigeria (Kim *et. al*., 1986, Kassam *et. al*., 1975). The soil type is a lixisol with high sand content (FAO, 2003). The field had previously been depleted of nitrogen (N) by planting maize at high densities for two growing seasons without fertilizer application and removing the above ground biomass after each growing season. The experimental field was cleared, ploughed and harrowed. The physical and chemical properties of the field prior to land preparation in each cropping season, as well as precipitation during the growing season at the location are shown in Table 3.1.

3.2 Genetic materials used in the study

Fourteen normal endosperm tropical maize experimental and commercial hybrids were used in the study. The materials used were developed at the International Institute of Tropical Agriculture Ibadan. These hybrids (Table 3.2) had previously been shown to have contrasting responses under low N fertilizer application (Meseka, 2005). Oba Super-1 and Oba Super-2, the two commercial hybrids included in this study, have been marketed by seed companies in Nigeria since 1984; Oba Super-2 is N-efficient while Oba Super-1 is N-inefficient (Sanginga *et al*., 2003). The experiment was carried out in three consecutive years of 2006, 2007 and 2008.

3.3 Experimental design and procedures

The experiment was laid out in a randomized complete block design with four replications. The experimental field was divided into three blocks viz: high-N, low-N and no-N. The high-N block received 90 kg N/ha, the low-N block received 30 kg N/ha and the no-N block received 0 kg N/ha. The no-N block served as a control. The N fertilizer levels were separated from one another by at least 5.0 m to minimize

Table 3.1. Physical and chemical properties and precipitation at the experimental site

	Block								
	0 kg N/ha			30 kg N/ha			90 kg N/ha		
Properties	2007	2008	2009	2007	2008	2009	2007	2008	2009
pH (1:1 H_2O)	4.9	4.8	5.8	5.1	4.9	5.9	5.3	4.7	5.9
Organic Carbon (g/kg)	3.8	2.6	4.2	3.7	4.1	5.0	4.0	4.2	5.6
Total N (g/kg)	0.41	0.25	0.42	0.39	0.39	0.54	0.40	0.41	0.70
Available P (mg/kg)	13.35	7.70	5.60	15.66	15.3	6.21	14.75	2.60	6.55
K (cmol/kg)	0.21	0.18	0.23	0.23	0.25	0.18	0.25	0.16	0.17
CEC (mol_c/kg)	3.86	3.77	2.97	4.12	3.15	1.75	4.42	3.42	1.31
Mechanical analyses									
Sand (g/kg)	680	700	740	680	700	820	660	680	820
Silt (g/kg)	150	140	120	140	130	60	160	150	60
Clay (g/kg)	170	160	140	180	170	120	180	170	120
Textural class (USDA)*	SL	SL	SL	SL	SL	LS	SL	SL	LS
Total precipitation (mm) (May to October)									
2006	880								
2007	1222								
2008	1379								

* SL: sandy loam; LS: loamy sand

Table 3.2. List of maize hybrids used in this study

S/N	Hybrid	Kernel colour	Previous performance for grain yield under low N*
E1	4001/4008	Yellow	High
E2	KU1409/4008	Yellow	High
E3	9450/MOK Pion-Y-S4	Yellow	Low
E4	KU1409/9613	Yellow	High
E5	4008/1808	Yellow	High
E6	4008/9071	Yellow	High
E7	9613/9006	Yellow	Low
E8	4058/Fun. 47-3	White	High
E9	1824/9432	Yellow	High
E10	4058/GH24	White	Low
E11	9071/4058	White	Low
E12	9006/4058	White	Low
E13	OBA SUPER-1 (commercial hybrid)	White	Low
E14	OBA SUPER-2 (commercial hybrid)	Yellow	High

* Source: Meseka (2005)

N movement from one treatment to the other. The N treatments were therefore kept as separate blocks. The same randomization with respect to hybrids was used in each N treatment. In each block (for each N level), plots consisted of four rows of 5.0 m length. Row and hill spacing were 0.75 and 0.25 m, respectively. Two seeds were planted per hill and later thinned to one to obtain a plant population density of 53,333 plants per hectare. One of the two inner rows in each plot was used for yield determination, while the other rows were used for destructive sampling. At planting, P in the form of single super phosphate and K as muriate of potash were applied at the rate of 60 kg P_2O_5/ha and 30 kg K_2O/ha respectively (Adeoye and Agboola, 1985). N fertilizer in the form of urea was applied in two equal split doses, the first half at 2 weeks after planting (WAP) and the second dose at 4 WAP. Herbicides complemented with hand weeding were used to achieve complete weed control.

3.4 Data collection

3.4.1 Agronomic and yield traits

Agronomic and yield data collected are as described below:

- Days to anthesis and days to silking: number of days from planting to when 50% of plants in a plot shed pollen or show silk extrusion.
- Anthesis – silking interval: derived as the difference in days between days to anthesis and days to silking.
- Ear leaf chlorophyll concentration: measured at silking on the middle of the upper ear leaf (Chapman and Barreto, 1997) of five competitive plants per plot using a portable Single-Photon Avalanche Diode (SPAD-502) chlorophyll meter (Minolta, 1989)
- Plant and ear heights: measured in meters on five competitive plants as distance from the ground level to the collar of the upper most leaf and upper ear leaf respectively
- Ears per plot: number of ears harvested in a plot.
- Ear weight per plot: measured in kg as the weight of all ears harvested in a plot.
- Ear aspect: scored on a scale of 1 to 5 where 1 = clean, uniform, large and well-filled ears and 5 = ears with undesirable characters such as incomplete filling and evidence of insect pest damage or fungal disease.
- Kernel rows: average number of kernel rows measured on 5 top ears

- Number of kernels: number of kernels on ear per plant after shelling. Estimated indirectly through the relationship between weight of 200 kernels, weight of total number of kernels and number of ears harvested per plot.
- Grain moisture at harvest: measured with a portable Dickey-John moisture tester (Model 14998, Dickey-John Corporation, Auburn USA) as the percent moisture content of shelled kernels.
- Kernel weight: measured in g as the weight of 1000 kernels adjusted to 15% moisture content. Estimated by weighing a representative 200 kernels and multiplying by 5.
- Grain yield: measured in Mg/ha adjusted to 15% moisture content. All ears in the unsampled row of a plot were harvested and shelled and the fresh weight, percent moisture content of shelled grains and number of plants at harvest used to estimate grain yield.
- Number of plants per plot: the number of plants on the unsampled row of each plot at harvest

All data with the exception of days to anthesis, days to silking, and anthesis-silking interval were collected on the row reserved for grain yield in each plot.

3.4.2 Plant material for N evaluation

For each N treatment, four representative plants were harvested by cutting near the soil surface at the silking date (R1) of each hybrid and after plants had reached physiological maturity (R6) to determine total biomass yield. All plant stover at R1 and stover (with ears removed) at R6 were chopped and dried in a forced-draft oven (60^{o}C) for 72 hours. The harvested ears were also oven-dried at 60^{o}C for 72 hours. The dry weight of each sample was determined after drying to constant weight. At R1, plant biomass (g/plant) was calculated by dividing the dry weight of each sample by four; while at R6 total plant biomass (g/plant) was calculated from the average of the sum of ear and stover weights. Post-silking biomass accumulation (g/plant) was calculated by subtracting plant biomass at silking from plant biomass at maturity. Individual samples were ground, passed through 20-mesh screen and stored in the refrigerator for assay. Grain and stover sub-samples were analyzed for total N concentration (g/kg) at the University of Illinois, Urbana-Champaign using a combustion technique (NA2000 N-Protein, Fisons Instruments. N content (g/plant) of stover at R1 was calculated by multiplying the N concentration by the dry weight.

Total N content (g/plant) at R6 was calculated by multiplying the N concentration for the stover and grain samples by their respective dry weights and summing both values. N-remobilization and post-silking N-accumulation were estimated adopting the balance method, by assuming that all N absorbed after silking was allocated to the grain. Therefore, N-remobilization was calculated as stover N content at silking minus stover N content at harvest. Post-silking N-accumulation which represents the quantity of total N at harvest absorbed after silking and was calculated as N content at harvest minus N content at silking.

Data from grain yield and plant N content were used to calculate efficiencies of N-use (NUE, kg grain /kg fertilizer N), N-uptake (NupE, kg plant N /kg fertilizer N) and N-utilization (NutE, kg grain/ kg plant N) following the procedure of Craswell and Goodwin (1984) as shown in equations (1) – (3) below. Nitrogen harvest index (NHI) was calculated as the ratio of the N content of grains to total N content of above-ground dry matter. Protein content was estimated by multiplying grain N content by 6.25 (Bender, 2005).

$$\mathrm{NUE} = \frac{\mathrm{GYF} - \mathrm{GYU}}{\mathrm{NRF}} \times 1000 \text{ (kg grain /kg supplied N)} \quad (1)$$

$$\mathrm{NupE} = \frac{\mathrm{NCF} - \mathrm{NCU}}{\mathrm{NRF}} \text{ (g plant N /g supplied N)} \quad (2)$$

$$\mathrm{NutE} = \frac{\mathrm{GYF} - \mathrm{GYU}}{\mathrm{NCF} - \mathrm{NCU}} \times 1000 \text{ (g grain /g plant N)} \quad (3)$$

Where GYF and GYU represent the grain yield (Mg/ha) in fertilized and unfertilized plots; NRF is the N fertilizer rate (kg/ha); NCF and NCU represent total plant N content (kg/ha) in fertilized and unfertilized plots.

3.4.3 Plant material for RNA extraction

Three immature ear shoots per plot were covered with shoot bags to prevent pollination. The shoots were harvested at silking. The husk and silk were removed and a pooled allometric 2 cm sample of the shoots quickly minced in a clean petri dish to enhance penetration of stabilization solution and immediately preserved inside a centrifuge tube in RNA*Later*® stabilization reagent (Ambion Inc., Austin, TX). Samples were later stored at -80^{o}C until used for RNA isolation.

3.4.4. Microarray procedure

For the microarray experiment, four hybrids: KU 1409/9613 (E4), KU 1409/4008 (E2), 4001/4008 (E1) and Oba Super-1 (E13) were chosen based on their performance under low-N in 2006 and 2007. The hybrid Oba Super-1 (E13) used as reference had previously been shown to be N-use inefficient.

3.4.4.1 RNA isolation

Preserved frozen earshoot tissues were homogenized in liquid nitrogen in a mortar and pestle (Coors Tek 60319). Total RNA from homogenized earshoot tissues was isolated using TRIzol® reagent according to manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Briefly, ground tissue was suspended in TRIzol with a TRIzol to tissue ratio of 1 ml/100 mg and homogenized for 5 minutes at room temperature. Chloroform (20% volume of TRIzol volume used) was added to the homogenate, shaken vigorously and incubated at room temperature for 3 minutes. This was followed by centrifugation at 12000 x g for 15 minutes at 4^{o}C. Isopropyl alcohol (50% volume of TRIzol used) was used to precipitate RNA from the aqueous phase in a fresh tube. After incubation at room temperature for 10 minutes, the sample was centrifuged at 12000 x g for 10 minutes at 4^{o} C. The RNA, which appears as a gel-like pellet on the side and bottom of the tube was washed by removing the supernatant and adding 75% ethyl alcohol (100% volume of TRIzol used). The sample was mixed by vortexing and centrifuged at 7500 x g for 5 minutes at 4^{o} C. After the removal of the supernatant, the RNA was air dried for about 10 minutes and then dissolved in 80 µl RNase-free water. The isolated RNA was DNase I (RNase free) treated to eliminate genomic DNA contamination and purified using the RNeasy® Plant Mini Kit (Qiagen, Chatswort, CA, USA) following manufacturer's guidelines. Purified RNA samples were quantitatively assessed and qualified using the Nanodrop® ND-1000 UV-visible spectrophotometer (Wilmington, DE, USA) and 1.0% agarose gel electrophoresis analyses, respectively.

3.4.4.2 Target labeling and array hybridization

Labelling reactions were performed following the indirect labeling procedure of Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion Inc., 2006) modified for half reaction. 1.5 µg of total RNA was used as template for the synthesis of complementary RNA (cRNA). Briefly, first strand cDNA from each sample was synthesized by reverse transcription (RT) from 1.5 µg of total RNA using T7

Oligo(dT) as primer following manufacturer's instructions. Second strand cDNA was synthesized using DNA polymerase and RNase H. The second strand of cDNA was purified and amino allyl labeled (aRNA) by in vitro transcription (IVT) using 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (aaUTP) and T7 enzyme mix. The labeled aRNA was thereafter purified and quantified using Nanodrop® ND-1000 UV-visible spectrophotometer (Wilmington, DE, USA). Approximately 20 – 40 µg of amplified RNA (aRNA) was obtained. For each sample 6.0 µg of the amino allyl aRNA was vacuum dried, re-suspended in Coupling Buffer and labeled with either Cy3 or Cy5 Mono NHS Ester (CyDye™ Post-Labeling Reactive Dye Pack, Amersham). After purification, incorporation of Cy3 and Cy5 was measured on Nanodrop® ND-1000 UV-visible spectrophotometer (Wilmington, DE, USA).

Microarray hybridization of samples and reference to the Arizona array slides was performed manually using the Corning hybridization cassettes following manufacturer's instructions. Briefly, the array slides were UV cross-linked at 1800 µJoules in a Stratalinke (Stratagene), washed in 1% SDS for 5 minutes with strong up and down agitation, rinsed in reagent grade water (by dipping two times each in five different containers) and 100% ethyl alcohol (by dipping ten times) and drained by spinning in a centrifuge at 1000 rpm for 1 minute. Array slides were subsequently pre-hybridized for 45 minutes at 42^{o} C in a pre-hybridization buffer containing 5X SSC, 0.1% SDS, and 0.01 g/ml BSA. After pre-hybridization, slides were washed in reagent grade water (by dipping two times each in five different containers) and 100% isopropyl alcohol (by dipping five times) and immediately drained by centrifugation at 1000 rpm for 1 minute. For each hybridization, equal amounts of samples and reference to be hybridized on the same array (Cy3 and Cy5) were combined in 0.2 ml microcentrifuge tube, mixed and vacuum dried in the dark until less than 1.0 µl was left. A total of four replications were made (two biological replicates with each dye-swapped). The dried probes were re-suspended in 130 µl hybridization buffer containing 5X SSC, 0.1% SDS, 500 µl/ml formamide, 40 µl/ml yeast tRNA and 20 µl/ml salmon sperm DNA. This mixture was heated for 3 minutes at 95^{o} C in a PCR thermal cycler, iced for 30 seconds and particles removed by spinning for 2 minutes. 50 µl of the pre-heated sample was applied to array slides covered with cover slips (Lifterslips, Erie Scientific) in hybridization cassette following manufacturer's guidelines. Hybridization was done in a water bath maintained at 42^{o} C for about 16 hours (overnight).

After hybridization, arrays were subjected to a four step washing in the dark and with rotation in 2X SSC, 0.1% SDS warmed to 42^{o} C for 5 minutes, 2X SSC for 10 minutes at room temperature, 0.2X SSC for 10 minutes at room temperature, 0.2X SSC for 5 – 10 minutes at room temperature. Immediately after the fourth wash, arrays were drained by centrifugation at 4000 rpm for 1 minute.

3.4.4.3 Generation of expression values and data analysis

Slides were scanned with GenePix 4000B scanner (Axon Instruments) immediately after drying at 10 µm resolution and 100% laser power. Different PMT (Photo-Multiplier Tube) values were used to achieve good signal intensities for the majority of spots and to minimize the number of spots on the array with saturated signal values. The signal intensity of the two channels was normalized by adjusting the PMT settings such that the signal ratio of Cy5/Cy3 across the whole array was approximately 1.0.

3.4.4.4 Data collection, normalization and analysis

Spot intensities were quantified using Axon Genepix Pro 6.0 image analysis software (Axon Instruments) and channel ratios were determined by the median-of-ratio method. The data sets were filtered for spots flagged as 'Bad' or 'Not Found' by Genepix Pro 6.0. Raw data from Genepix were then imported into Bioconductor R and analysed using the LIMMA (Linear Models for Microarray Data) library (Smyth, 2004; software manual available from http://bioinf.wehi.edu.au/limma/). Spots below background, or flagged as "bad" or "Not found" by Genepix Pro, for which spot fluorescence cannot be distinguished from background were ignored in all pre-processing and analysis steps. After background subtraction from each array spot, spot quality weighing with good spots of 1.0 and flagged spots of 0.1 was carried out in the LIMMA software package. Within-array normalization of the expression values was done using global loess, and between arrays using scale by the LIMMA package from Bioconductor. The normalized data for each slide/dye combination were median-centered to ensure comparable expression measures across slides. Linear model fit was computed using the least square method. Differentially expressed genes are classified as significant when they have a p value ≤ 0.01.

3.5 Validation of expression profiles using Real time RT-qPCR

Twelve genes were selected from the microarray result for qRT-PCR analyses.

The RNAs extracted for the microarrays were also used for real-time RT-PCR to validate the microarray results. First strand cDNA was synthesized using the SuperScript^{TM} III Reverse Transcriptase kit (www.invitrogen.com) on 1.0 µg of total RNA and oligo d(T) as primer following the procedure outlined by the manufacturer. The synthesized cDNA was subjected to a 20-fold dilution before use as template for real time RT-PCR. PerfeCTa SYBR Green FastMix, Rox master mix (Quanta Biosciences) was used as reaction cocktail for qRT-PCR following manufacturer's instructions. Briefly, a 20 µl total volume reaction contained 3.0 µl diluted cDNA, 10 µl SYBR Green FastMix, Rox master mix, 1.0 µl of 10 mM dNTP Mix, and 1.0 µl each of 12 mM gene-specific forward and reverse primers. The working concentrations of the cDNA products were optimized for the housekeeping gene glyceraldehyde-phosphate-dehydrogenase (GAPDH) based on the log2 of the resulting cycle threshold (CT) values. All reactions were subjected to a single-step 40 PCR cycling (15s 95^{o} C, 30s 60^{o} C) followed by a standard melting curve, after the initial 3 minutes denaturation at 95^{o} C using the MJ Opticon II thermocycler (www.bio-rad.com). Two biological replicates of each sample were used and replicated twice on the PCR plate. Primer sequences for real-time RT-PCR were designed using the Vector NTI software with amplicon length between 150 and 250 bp (Appendix 4). The primers were synthesized by Invitrogen (www.invitrogen.com). Normalization of amplification curves was carried out with the baseline subtraction in accordance with the BioRad software package (MJ Opticon Monitor Analysis Software version 3.1). The CT value was set according to points where the efficiencies of all reactions were closest to 100%, and the CT values for each assay exported to Excel for comparison.

Relative expression of selected genes was calculated using the ΔΔCT procedure (Livak and Schmittgen, 2001) on means of technical and biological replications GAPDH

as housekeeping gene and the hybrid Oba Super-1 as reference.

3.6 Statistical Analyses.

Analysis of variance (ANOVA) for a Randomized Complete Block was carried out on agronomic and physiological traits using the Proc GLM procedure from SAS statistical software (SAS Institute Inc., 2003). Individual analyses were performed for the years and each N rate and thereafter for the years and N rates combined. In both cases, all effects were considered fixed.

To understand how the plant functions at different N-input levels, phenotypic correlations were carried out on standardized data among genotypic means, between pairs of agronomic and physiological traits. To determine the associations between hybrid performance under low N and high N conditions, linear correlation analysis was also conducted between variables measured under both levels of N. Path coefficient analysis was carried out to capture the intricate relationships among selected physiological and yield traits significantly related to kernel number, NUE and grain yield (Li, 1975; Williams and Demment, 1990). Based on the diagram of causation (Figure 3.1), multiple regression analysis was used to calculate the Path coefficients (partial regression coefficients) for selected physiological and yield traits and its effect on grain yield in SAS. Path coefficient analysis is a powerful and flexible statistical tool used to examine the relationships between measured variables. It measures the direct influence of one variable upon the other and permits the separation of the correlation coefficients into components of direct and indirect effects (Dewey and Lu, 1959). Indirect effects were calculated by multiplying the respective correlation coefficients between the two traits by the standardized path coefficients of the indirectly related trait. The use of the standardized path coefficients eliminates the effect of scale of measurement having been generated from data standardized to assume a normal distribution with a mean of zero and standard deviation of one. The total correlation between any causal trait and a dependent trait was determined by summing the path coefficient between the two variables with the indirect effects of the other causal variables on the dependent variable.

Low N index (LI) was computed from mean grain yield of hybrids following the procedure of Chantachume *et al*. (1998) as:

$$\mathrm{LI} = \frac{\mathrm{X1}}{\mathrm{X2}} \div \frac{\mathrm{Y1}}{\mathrm{Y2}}$$

Where

X1 = mean of grain yield of hybrid X under low-N;

X2 = mean of grain yield of hybrid X under high-N

Y1 = mean of grain yield of all hybrids in low-N environment

Y2 = mean of grain yield of all hybrids in high-N environment

LI > 1 indicates tolerance to low N while LI < 1 is indicative of susceptibility to low N.

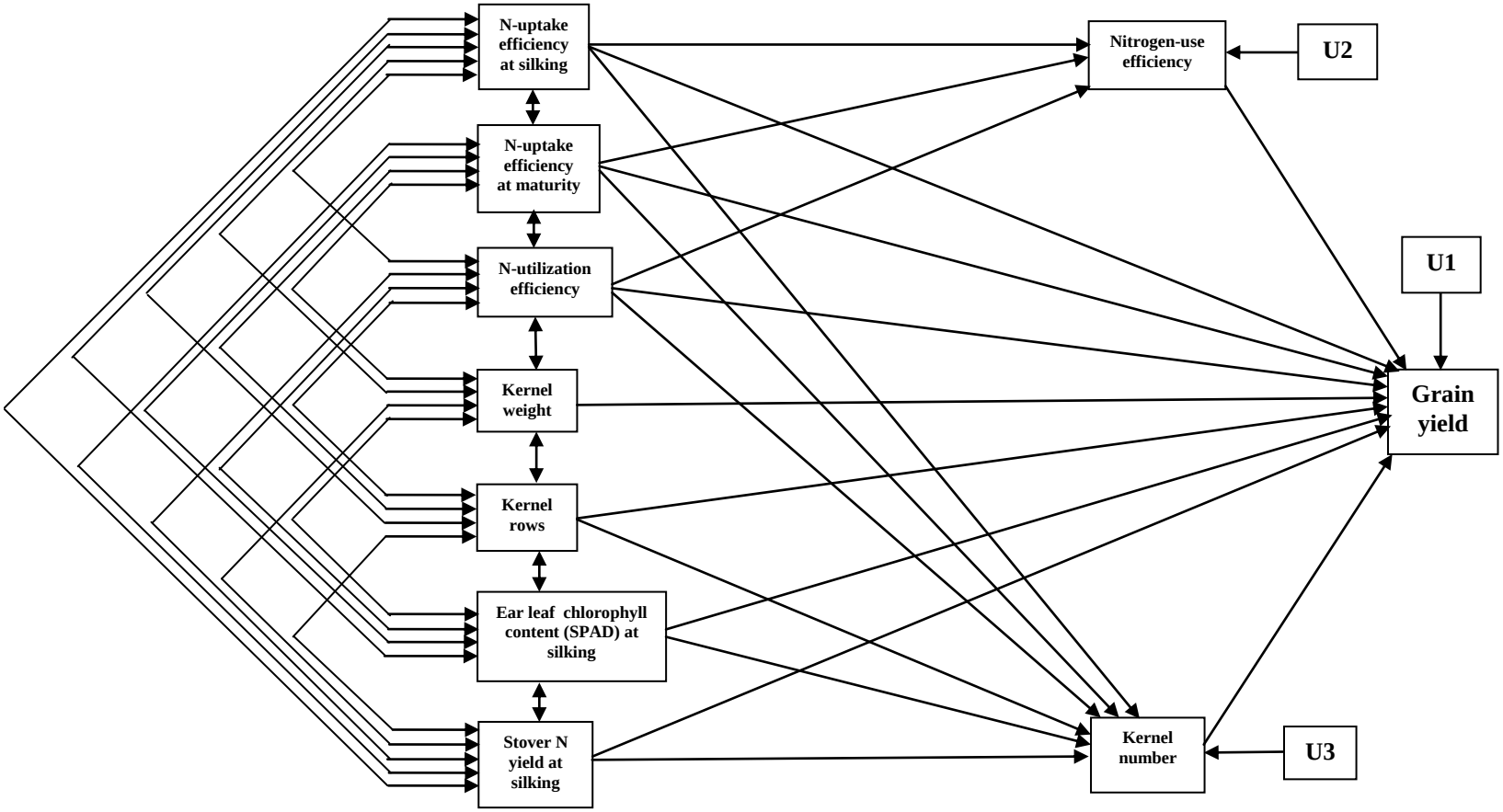

Figure 3.1. Path diagram of the causal model indicating the relationships between Grain yield and selected physio-morphological and yield traits. U1, U2 and U3 are the unexplained variances associated with Grain yield, N-use efficiency and Kernel number respectively.

CHAPTER 4
RESULTS

4.1 Performance of hybrids

4.1.1 Agronomic traits

There were significant ($p < 0.01$) differences among the genotypes studied for grain yield and its components at high-N, low-N and no-N. In the analysis of variance combined across years and N levels, the proportion of the mean square accounted for by genotype for grain yield and its components was much higher than the proportion of the mean square for the interaction terms involving genotype. Genotype × nitrogen as well as genotype × nitrogen × year interactions were significant ($p < 0.01$) for number of kernels, ears per plant, one thousand kernel weight and grain yield. While the mean square of genotype × nitrogen interaction for ears per plant was about two-fifth that of genotype, this interaction was over four times less than the corresponding mean square of genotype for number of kernels, one thousand kernel weight and grain yield. Differences among the genotypes for plant and ear heights, as well as flowering traits, except anthesis-silking interval (ASI), were also significant ($p < 0.01$) at high-N and low-N. Under no-N, significant ($p < 0.05$) differences among genotypes were observed for these traits, except for days to anthesis. In the combined analysis over the years and N levels, genotype × nitrogen interaction effect for these traits was not significant (Table 4.1).

Nitrogen stress depressed grain yield and yield components with the effect being more pronounced under no-N (Table 4.2). Mean grain yield averaged across years, was reduced by 35.2% at low-N and 76.4% at no-N. Of the grain yield components, number of kernels was the most affected by N stress showing a 21.5% and 60.3% reduction at low-N and no-N respectively. Kernel weight was reduced by 2.4% at low-N and 16.7% at no-N. Number of ears per plant was less than one even under high-N (0.95) conditions. Kernel rows averaged 14.3 at high-N, 13.6 at low-N and 12.1 at no-N. Estimated from number of kernels and kernel rows, number of kernel per row was 11.0, 19.3 and 23.4 at no-N, low- N and high-N respectively. These values indicate 17.9% and 53.4% reduction in number of kernels per row at

Table 4.1. Analysis of variance of agronomic traits at each of three N levels and for the combined N levels analysis in 14 tropical maize hybrids in Mokwa, Nigeria from 2006 to 2008.

N level	Source of variation	Days to anthesis (×10)	Days to silk (×10)	ASI (days)	Plant height (m) ($\times10^{-2}$)	Ear height (m) ($\times10^{-2}$)	Kernel rows (×10)	Number of kernels ($\times10^{4}$)	Ear aspect (scale of 1 - 5)	Ears per plant ($\times10^{-2}$)	One thousand kernel weight (g) ($\times10^{3}$)	Grain yield (Mg/ha)
no-N	Y	56.90***	45.39***	48.29***	345.75***	83.23***	3.48***	12.82***	3.14***	46.18***	55.41***	21.45***
	H	0.44ns	0.61**	1.78*	8.13**	2.53**	0.37***	0.57**	1.78***	9.24***	2.87***	0.92***
	HxY	0.72***	0.57**	1.73*	6.21**	1.59ns	0.11ns	0.43*	0.44ns	4.63***	1.28***	0.65***
low-N	Y	52.87***	50.91***	22.60***	224.65***	90.33***	2.08***	24.78***	2.43***	43.30***	12.55***	62.93***
	H	2.21***	2.27***	1.66ns	7.86***	3.35**	1.23***	1.14***	1.15***	5.46***	2.45***	1.55***
	HxY	0.82**	0.96**	1.32ns	5.53***	1.92*	0.21***	0.60***	0.38***	2.14*	0.81***	0.75***
high-N	Y	23.41***	17.32***	12.79***	576.46***	280.57***	0.50**	53.36***	13.42***	53.62***	33.03***	151.02***
	H	1.10***	0.78**	1.42ns	8.08***	3.72***	1.30***	1.15***	0.51**	3.23*	3.59***	1.18***
	HxY	0.35ns	0.42ns	0.96ns	4.72***	1.34ns	0.15**	0.58*	0.53***	3.47***	0.91***	1.78***
Combined N levels analysis	Y	121.46***	101.90***	76.97***	980.83***	381.44***	4.04***	71.81***	10.82***	118.96***	51.26***	190.74***
	N	119.58***	198.38***	98.91***	1554.07***	486.69***	19.78***	175.75***	4.12***	439.32***	67.18***	401.06***
	H	3.01***	2.65***	3.06***	19.81***	7.46***	2.59***	1.60***	2.60***	9.09***	7.41***	2.73***
	YxN	5.86***	5.86***	3.35**	83.02***	36.35***	1.01***	9.58***	4.09***	12.06***	24.87***	22.33***
	HxY	1.12***	1.31***	1.80**	10.80***	2.26**	0.33***	0.78***	0.79***	5.24***	1.42***	0.99***
	HxN	0.37ns	0.50ns	0.90ns	2.12ns	1.07ns	0.16**	0.63***	0.41*	4.42***	0.75**	0.46***
	HxNxY	0.38ns	0.32ns	1.10ns	2.83ns	1.30ns	0.08ns	0.42**	0.28ns	2.50**	0.79***	1.10***

*, **, ***: significant at 0.05, 0.01, 0.001 probability levels, respectively; ns: not significant; Y = year, N = N level, H = genotype.

Table 4.2. Effects of nitrogen fertilizer application on agronomic trait means of 14 tropical maize hybrids grown under 0 (no-N), 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa Nigeria between 2006 and 2008.

	N-rate					
Traits	no-N mean	low-N mean	high-N mean	LSD (0.05)	% reduction at no-N	% reduction at low-N
Days to anthesis	60.5	57.2	55.3	0.39	-9.4	-3.4
Days to silk	64.1	59.6	57.4	0.41	-11.7	-3.9
Anthesis-silking interval (days)	3.6	2.5	2.1	0.21	-71.4	-19.0
Plant height (m)	1.27	1.77	1.83	0.03	30.6	3.3
Ear height (m)	0.55	0.82	0.86	0.02	36.0	4.7
Kernel rows	12.1	13.6	14.3	0.19	15.4	4.9
Number of kernels	132.7	262.6	334.5	11.13	60.3	21.5
Ears per plant	0.64	0.85	0.95	0.03	32.6	10.5
Ear aspect (scored on a scale of 1 - 5)	3.14	2.83	2.96	0.10	-6.1	4.4
One thousand kernel weight (g)	184.2	215.8	221.2	4.07	16.7	2.4
Grain yield (Mg/ha)	0.95	2.61	4.03	0.07	76.4	35.2

low-N and no-N respectively. Under low-N, number of days to silking was delayed by 2.3 days while anthesis was delayed by 1.9 days. ASI was not adversely affected by N stress (2.1 days at high-N, 2.5 days at low-N and 3.5 days at no-N). Severe N stress at no-N reduced plant height and ear height by 30% and 36%, respectively. Under low-N, reductions in these traits were 3.3% and 4.7%, respectively.

At high-N, the range in mean grain yield across the years among hybrids varied from 3.3 Mg/ha to 4.4 Mg/ha and from 2.0 Mg/ha to 3.2 Mg/ha at low-N. The range under no-N was 0.5 Mg/ha to 1.5 Mg/ha, which translates to a reduction of 64 - 87%. Hybrids 4001/4008 and KU1409/9613 produced the highest yields at high-N and low-N, respectively. The hybrid Oba-Super-1 was the lowest yielding under both high-N and low-N application (Table 4.3).

Tolerance values of the hybrids to low-N, indicated by a low-N index, are presented in Table 4.3. Eight hybrids (4001/4008, KU1409/4008, KU1409/9613, 4008/1808, 4008/9071, 4058/Fun 47-4, 1824/9432 and Oba Super-2), showed tolerance to low-N while the remaining six were susceptible. Among the eight hybrids considered to be low-N tolerant, 4008/9071 had below average grain yield under high-N, while Oba Super-2 had below average grain yield at both high-N and low-N. To determine the N-use efficiency of hybrids, the performance of the hybrids under high-N was plotted against their performance under low-N. Six of the eight hybrids classified as low-N tolerant based on their low-N index values, were found to be efficient and responsive (Figure 4.1) and these are 4001/4008 (E1), KU1409/4008 (E2), KU1409/9613 (E4), 4008/1808 (E5), 4058/Fun 47-4 (E8), 1824/9432 (E9). The remaining two, namely: 4008/9071 (E6) was efficient but non-responsive and Oba Super-2 (E14) was found to be inefficient and non-responsive. Two N-susceptible hybrids (9071/4058 (E11), 9006/4058 (E12)) had comparable yields to the low-N tolerant, efficient and responsive hybrids (E1, E2, E4, E5, E8, E9) under high-N, hence responsive to N application. These results suggest that hybrids E11 and E12 have specific adaptation to the high-N environment. Overall, hybrid E1 and E4 were the most efficient and responsive hybrids, while Oba Super-1 (E13) was the most in-efficient and non-responsive hybrid.

4.1.2 Physiological traits

Differences among genotypes for ear leaf chlorophyll concentration (SPAD) and all traits associated with biomass production were significant ($p < 0.05$) at the three

Table 4.3. Means of grain yield and yield components of 14 tropical maize hybrids grown under 0, 30 and 90 kg N/ha fertilizer application at Mokwa, Nigeria between 2006 and 2008.

HYBRIDS	Ears per plant			Kernel rows			Number of kernels			One thousand kernel weight (g)			Grain yield (Mg/ha)			Low-N index
	0	30	90	0	30	90	0	30	90	0	30	90	0	30	90	
4001/4008	0.74	0.90	0.95	12.7	14.3	15.2	153.0	319.2	357.0	184.8	215.3	222.2	1.29	3.09	4.42	1.2
KU 1409/4008	0.63	0.77	0.92	11.7	12.5	13.3	163.9	294.4	335.5	207.9	238.5	253.5	1.53	2.93	4.21	1.0
9450/MOK Pion-Y-S4	0.58	0.89	1.04	10.8	11.8	11.9	127.9	241.2	281.0	153.3	191.4	197.7	0.48	2.29	3.68	0.9
KU 1409/9613	0.62	0.88	1.00	12.4	14.3	14.5	145.2	300.1	363.5	199.9	226.8	235.6	0.98	3.18	4.30	1.1
4008/1808	0.82	0.91	0.98	11.8	12.1	13.1	128.5	229.9	318.6	203.6	245.8	242.1	1.10	2.80	4.18	1.0
4008/9071	0.61	0.85	0.91	12.1	13.3	14.2	143.4	274.3	318.9	199.7	218.6	213.7	1.00	2.75	3.70	1.3
9613/9006	0.48	0.83	0.95	11.7	13.6	13.7	136.7	249.7	303.9	178.7	206.3	232.6	0.77	2.46	3.90	0.9
4058/Fun 47-4	0.66	0.86	0.90	12.7	14.4	15.1	130.3	266.2	367.6	179.1	212.4	212.1	1.04	2.73	4.25	1.0
1824/9432	0.64	0.94	0.95	12.9	15.3	15.4	119.8	286.8	368.3	179.7	207.0	219.0	0.89	2.95	4.24	1.1
4058/GH 24	0.72	0.91	0.93	12.5	14.1	15.0	102.2	240.7	358.5	178.8	207.9	212.5	0.68	2.33	3.83	0.9
9071/4058	0.52	0.71	0.90	12.4	14.7	15.6	116.5	284.9	379.0	170.2	201.8	190.0	0.83	2.23	4.28	0.8
9006/4058	0.60	0.78	1.07	12.0	13.3	13.6	166.5	229.6	299.8	173.1	221.1	236.5	0.86	2.27	4.27	0.8
OBA SUPER-1	0.61	0.78	0.94	12.0	13.3	14.5	88.7	221.1	308.8	172.5	218.9	213.4	0.63	2.02	3.34	0.9
OBA SUPER-2	0.67	0.87	0.92	12.2	13.9	14.6	135.3	238.3	323.2	197.2	209.9	215.6	1.17	2.47	3.90	1.0
Mean	0.64	0.85	0.95	12.1	13.6	14.3	132.7	262.6	334.5	184.2	215.8	221.2	0.95	2.61	4.03	
CV	21.7	13.3	12.6	7.90	5.12	6.05	36.7	18.9	16.6	12.2	7.32	8.19	17.2	13.2	11.4	
LSD (0.05)	0.11	0.09	0.10	0.78	0.56	0.70	39.3	40.1	44.8	18.1	12.6	14.67	0.13	0.28	0.37	

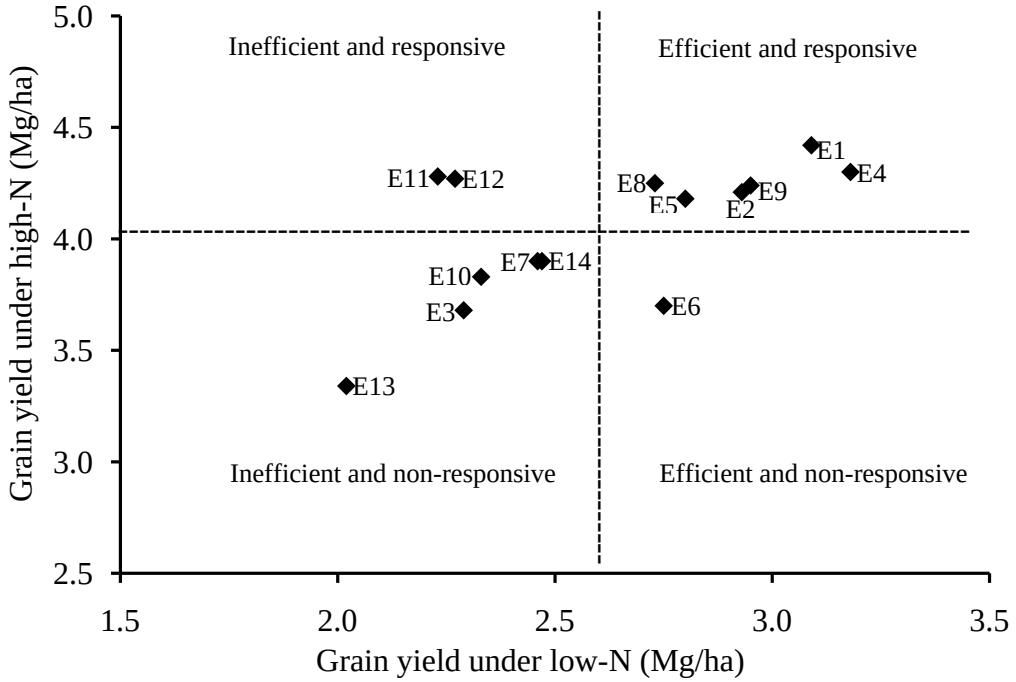

Figure 4.1. Biplot for grain yields of 14 tropical maize hybrids at high and low levels of applied nitrogen fertilizer. Broken lines represent mean grain yields.

levels of N application. In the combined analysis, differences among genotypes for these traits were also significant ($p < 0.01$). Genotype × nitrogen interaction effect was not significant for ear leaf chlorophyll concentration (Table 4.4). For all the (stover and grain) N content traits, at the two stages of sampling, genotype and genotype × nitrogen interaction effects were significant ($p < 0.01$). Similar results were obtained for the post-silking N accumulation traits. (Table 4.5).

Ear leaf chlorophyll concentration (SPAD) showed more pronounced variation among hybrids at no-N than at low-N and high-N. N stress reduced Ear leaf chlorophyll concentration by 22.5% at low-N and 52.7% at no-N. Under low-N, stover dry weight by 3.5% at silking and 20.7% at maturity. Although N stress reduced grain weight per plant by 33.4% at low-N and 75.4% at no-N, the reduction in harvest index (HI) was 9.3% and 23.3% at low-N and no-N respectively. This indicates a relative stability in the proportion of percent reductions in grain weight to HI at low-N and no-N. Post-silking dry matter accumulation was reduced by 46.7% at low-N and it contributed 52.7%, 37.3% and 34.7% to the total dry matter production at maturity under high-N, low-N and no-N, respectively. A 2% stover remobilization was observed under low-N (Table 4.6).

Nitrogen stress reduced stover N concentration by 33.2% at silking and 44.9% maturity under low-N (Table 4.7). At low-N, the reduction in stover N content at silking was almost the same as its corresponding reduction in N concentration (34.5%). The stover N content at maturity suffered a 50.7% reduction at low-N and 73.9% reduction at no-N. Nitrogen stress under low-N reduced grain N concentration by 15.9%, Grain N content by 42.1% and total N content at maturity by 45.4%. Corresponding reductions at no-N were 8.2%, 77.2% and 76.0%, respectively. At low-N, 72% of the total N content at maturity was accumulated at silking while 60.1% of the total N content at maturity was accumulated at silking under high-N. Post-silking stover N accumulation was 7.3% and 75.6% higher at low-N and no-N, respectively, than at high-N. Compared to high-N treatment, Total N accumulation between silking and maturity was at low-N reduced by 61.6%. The stover N remobilized to meet kernel demand was 52.8% at low-N and 37.3% at high-N.

Total above ground dry weight varied from 145.7 to 191.1 g/plant (mean = 169.9 g/plant) under high-N, from 110.0 to 151.6 g/plant (mean = 125.3 g/plant) under low-N and from 39.9 to 75.3 g/plant (mean = 53.4 g/plant) under no-N. The highest above ground dry weight was produced by 4058/Fun 47-4 at high-N and KU1409/4008

Table 4.4. Analysis of variance of physiological traits at each of three N levels and for the combined N levels analysis in 14 tropical maize hybrids in Mokwa, Nigeria from 2006 to 2008.

N level	Source of variation	Stover dry weight at silking (g/plant) ($\times 10^2$)	Ear leaf chlorophyll (SPAD) ($\times 10$)	Stover dry weight at maturity (g/plant) ($\times 10^2$)	Grain weight (g/plant) ($\times 10^2$)	Total dry weight (g/plant) ($\times 10^3$)	Harvest index ($\times 10^{-3}$)	Stover dry matter accumulation (g/plant) ($\times 10^2$)	Total dry matter accumulation (g/plant) ($\times 10^3$)
no-N	Y	89.53***	70.69***	88.82***	29.16***	20.06***	155.81***	0.36ns	3.32***
	H	1.75***	4.23***	2.93***	3.10***	1.09***	21.41***	4.58***	1.32***
	HxY	1.29***	2.61***	1.47**	1.85***	0.50***	24.54***	1.11ns	0.39***
low-N	Y	153.47***	151.80***	139.57***	34.46***	31.25***	1.85ns	0.43ns	2.85***
	H	2.43***	3.63*	5.73***	2.71***	1.37***	6.25*	8.65***	1.65***
	HxY	2.54***	1.87ns	2.65***	1.81***	0.72***	3.51ns	4.01***	0.74***
high-N	Y	373.27***	408.46***	562.13***	284.19***	164.55***	6.73ns	21.25***	45.37***
	H	3.07***	3.37*	4.28***	6.45***	1.81***	6.06**	6.65***	2.30***
	HxY	4.70***	4.82***	8.98***	6.63***	2.56***	2.49ns	2.86***	1.31***
Combined N levels analysis	Y	473.54***	555.25***	569.20***	174.52***	137.41***	29.75***	4.40**	23.43***
	N	1090.68***	1926.67***	1610.41***	1311.89***	580.43***	447.23***	148.07***	214.17***
	H	2.83***	8.10***	7.81***	7.72***	2.78***	18.57***	10.31***	3.15***
	YxN	71.36***	37.85***	110.67***	86.65***	39.22***	67.32***	8.83***	14.05***
	HxY	4.62***	4.71***	7.02***	4.70***	1.88***	9.68***	4.13***	1.11***
	HxN	2.21***	1.57ns	2.57***	2.27***	0.75***	7.57**	4.78***	1.06***
	HxNxY	1.95***	2.29**	3.04***	2.80***	0.95***	10.42***	1.93***	0.67***

*, **, ***: significant at 0.05, 0.01, 0.001 probability levels, respectively; ns: not significant; Y = year, N = N level, H = genotype.

Table 4.5. Analysis of variance of N-related traits at each of three N levels and for the combined N levels analysis in 14 tropical maize hybrids in Mokwa, Nigeria from 2006 to 2008.

N level	Source of variation	Stover N content at silking (g/plant) ($\times 10^{-2}$)	Stover N content at maturity (g/plant) ($\times 10^{-2}$)	Grain N content (g/plant) ($\times 10^{-2}$)	Total N content (g/plant) ($\times 10^{-1}$)	Grain protein content (g/plant)	% N remobilization ($\times 10^{-2}$)	Stover N accumulation (g/plant) ($\times 10^{-2}$)	Total N accumulation (g/plant) ($\times 10^{-1}$)
no-N	Y	103.64***	23.94***	82.18***	15.56***	32.10***	77.85***	28.74***	4.09***
	H	1.81***	1.26***	4.68***	0.79***	1.83***	25.50***	2.52***	1.08***
	HxY	1.01***	0.42**	3.03***	0.39***	1.18***	5.07ns	0.85*	0.31***
low-N	Y	549.97***	14.59***	103.80***	19.54***	40.55***	102.25***	386.52***	8.97***
	H	5.10***	2.15***	5.34***	1.19***	2.09***	7.40***	7.96***	1.79***
	HxY	4.18***	1.05**	3.30***	0.65***	1.20***	3.46*	5.04***	0.84***
high-N	Y	1548.36***	208.22***	405.59***	35.36***	158.43***	1245.09***	2306.66***	86.85***
	H	8.33***	4.57***	13.29***	2.07***	5.19***	9.68***	16.86***	3.89***
	HxY	17.64***	8.22***	18.65***	4.02***	7.29***	5.47**	9.92***	1.96***
Combined N levels analysis	Y	1696.80***	89.13***	372.60***	61.88***	145.54***	709.33***	1604.88***	57.86***
	N	2834.88***	1151.32***	3237.05***	819.26***	1264.47***	264.50***	481.06***	150.96***
	H	6.75***	4.37***	12.68***	2.32***	4.95***	22.36***	14.88***	3.86***
	YxN	252.57***	78.81***	109.48***	4.29***	42.77***	357.92***	558.52***	21.03***
	HxY	11.81***	3.35***	11.47***	2.17***	4.48***	6.37*	7.49***	1.10***
	HxN	4.25***	1.80***	5.32***	0.86***	2.08***	10.11***	6.23***	1.45***
	HxNxY	5.51***	3.16***	6.75***	1.44***	2.64***	3.82ns	4.16***	1.01***

*, **, ***: significant at 0.05, 0.01, 0.001 probability levels, respectively; ns: not significant; Y = year, N = N level, H = genotype.

Table 4.6. Effects of nitrogen fertilizer application on and physiological trait means of 14 tropical maize hybrids grown under 0 (no-N), 30 (low-N) and 90 (high-N) kg N/ha measured at silking and maturity at Mokwa,Nigeria between 2006 and 2008.

	N-rate					
Measured variable	no-N mean	low-N mean	high-N mean	LSD (0.05)	% reduction at no-N	% reduction at low-N
At silking						
Stover dry weight (g/plant)	34.88	77.56	80.34	1.99	56.6	3.5
Ear leaf chlorophyll (SPAD)	19.18	31.40	40.52	0.79	52.7	22.5
At maturity						
Stover dry weight (g/plant)	35.18	76.02	95.91	1.92	63.3	20.7
Grain weight (g/plant)	18.24	49.29	74.01	1.78	75.4	33.4
Total dry weight (g/plant)	53.42	125.31	169.92	2.89	68.6	26.3
Harvest index	0.33	0.39	0.43	0.01	23.3	9.3
Post flowering changes						
Stover dry matter accumulation (g/plant)	0.29	-1.53	15.56	1.72	98.1	109.8
Total dry matter accumulation (g/plant)	18.53	47.76	89.57	2.57	79.3	46.7

Table 4.7. Effects of nitrogen fertilizer application on N related trait means of 14 maize hybrids grown under 0 (no-N), 30 (low-N) and 90 (high-N) kg N/ha measured at silking and maturity at Mokwa, Nigeria from 2006 to 2008.

	N-rate					
Measured variable	no-N mean	low-N mean	high-N mean	LSD (0.05)	% reduction at no-N	% reduction at low-N
At silking						
Stover N concentration (g/kg)	7.77	9.02	13.50	0.22	42.4	33.2
Stover N content (g/plant)	0.28	0.72	1.10	0.03	74.5	34.5
At maturity						
Stover N concentration (g/kg)	5.19	4.55	8.26	0.24	37.2	44.9
Grain N concentration (g/kg)	14.53	13.32	15.83	0.21	8.2	15.9
Stover N content (g/plant)	0.18	0.34	0.69	0.02	73.9	50.7
Grain N content (g/plant)	0.26	0.66	1.14	0.03	77.2	42.1
Total N content (g/plant)	0.44	1.00	1.83	0.03	76.0	45.4
Grain protein content (g/plant)	1.67	4.11	7.13	0.17	76.6	42.4
Post flowering changes						
Stover N accumulation (g/plant)	-0.10	-0.38	-0.41	0.03	75.6	7.3
Total N accumulation (g/plant)	0.16	0.28	0.73	0.04	78.1	61.6

at both low-N and no-N. Grain dry weight ranged from 59.4 to 85.2 g/plant (mean = 74.0 g/plant) under high-N, from 42.7 to 60.4 g/plant (mean = 49.3 g/plant) under low-N, and 10.5 to 29.8 g/plant (mean = 18.2 g/plant) at no-N. Differences in ear leaf chlorophyll concentration (SPAD) were more pronounced at no-N, but were similar under both high-N and low-N (Table 4.8). The differences in Stover N content measured at silking were more pronounced at high-N than at both low-N and no-N. Post-silking stover N accumulation ranged from -0.64 to -0.22 g/plant (mean = -0.41 g/plant) under high-N and -0.51 to -0.26 g/plant (mean = -0.38 g/plant) under low-N. KU1409/4008 accumulated the lowest stover N post-silking, but had the highest total N accumulation post-silking and total N content under the three N levels considered. This is an indication that KU1409/4008 possessed a high efficiency for N remobilization from stover to grain. The range in Grain N content was 0.88 to1.26 g/plant (mean = 1.14 g/plant) under high-N and 0.58 to 0.85 g/plant (mean = 0.66 g/plant) under low-N. KU1409/4008 had the highest Grain N content at low-N and no-N, which was significantly higher than that of other hybrids. Although 9613/9006 had the highest Grain N content at high-N, the value was not significantly different from that of KU1409/4008 (Table 4.9).

4.1.3 Efficiencies of N-uptake, N-utilization and N-use and Nitrogen harvest index

Genotype and genotype × N interaction effects were significant ($p < 0.01$) for N-use efficiency and its component traits at both high-N and low-N. Differences among the genotypes were not significant for Nitrogen harvest index (NHI) at low-N but were highly significant ($p < 0.01$) at high-N. Genotype × nitrogen interaction was not significant for NHI (Table 4.10).

N-uptake efficiency at silking and maturity were lower at high-N than at low-N. Compared to low-N, N-uptake efficiency was reduced by 60.4% at silking and by 20.7% at maturity. N-utilization efficiency and N-use efficiency were also reduced by 42.1% and 61.4% at high-N respectively. Nitrogen harvest index (NHI) was reduced by 6.5% at high-N suggesting a relative stability in this trait across N levels (Table 4.11). Thus, N-uptake efficiency, N-utilization efficiency, N-use efficiency and NHI reduced with increasing level of N application.

N-uptake efficiency, N-utilization efficiency, N-use efficiency and NHI decreased from low-N to high-N. While hybrids differed significantly ($p < 0.001$) for N-uptake efficiency, N-utilization efficiency, and N-use efficiency under high-N and

Table 4.8. Means of selected agro-morphological traits of 14 tropical maize hybrids at 0, 30 and 90 kg N/ha fertilizer application in Mokwa, Nigeria from 2006 to 2008.

HYBRIDS	Stover dry weight at silking (g/plant)			Stover dry weight at maturity (g/plant)			Grain weight (g/plant)			Total dry weight at maturity (g/plant)			Ear leaf chlorophyll at silking (SPAD)		
	0	30	90	0	30	90	0	30	90	0	30	90	0	30	90
4001/4008	30.6	81.0	81.1	42.6	82.4	98.0	24.0	54.4	77.5	66.6	136.8	175.6	21.8	34.5	41.1
KU 1409/4008	32.1	73.2	76.8	45.5	91.2	102.2	29.8	60.4	79.3	75.3	151.6	181.5	21.9	34.3	42.4
9450/MOK Pion-Y-S4	28.5	73.2	79.3	26.7	70.6	96.7	13.1	44.4	71.5	39.9	115.0	168.2	18.2	30.8	41.0
KU 1409/9613	35.0	86.9	79.3	34.2	79.2	94.2	23.1	45.5	78.8	57.2	124.6	173.0	20.3	33.1	42.2
4008/1808	35.0	80.1	79.5	38.7	77.5	93.4	21.3	52.1	76.0	59.9	129.7	169.4	20.2	33.2	41.4
4008/9071	44.5	73.6	91.2	37.2	79.8	86.0	20.0	42.7	59.4	57.2	122.5	145.4	20.5	30.6	38.9
9613/9006	36.4	78.7	78.9	31.8	71.8	95.5	16.3	46.5	73.6	48.1	118.4	169.2	17.6	31.5	38.4
4058/Fun 47-4	35.3	78.9	88.4	35.0	76.8	105.9	15.3	52.6	85.2	50.3	129.5	191.1	18.3	30.5	41.3
1824/9432	31.5	82.5	70.8	35.3	63.9	86.7	19.7	46.1	77.7	55.0	110.0	164.4	21.3	31.5	41.6
4058/GH 24	34.0	71.2	80.2	31.5	74.9	99.6	16.2	47.5	72.6	47.6	122.4	172.2	19.0	29.1	39.5
9071/4058	35.2	79.5	77.1	32.7	76.7	103.3	16.5	50.9	83.4	49.2	127.7	186.7	17.8	30.8	37.9
9006/4058	37.0	79.3	85.1	30.1	76.0	98.0	13.8	52.0	71.3	43.9	128.0	169.3	17.8	29.7	42.8
OBA SUPER-1	35.5	74.0	79.5	33.6	65.0	88.5	10.5	46.0	62.7	44.1	110.9	151.3	15.4	29.3	38.1
OBA SUPER-2	38.0	73.7	77.6	37.7	78.4	94.8	15.7	49.0	67.0	53.4	127.5	161.8	16.6	30.8	40.9
Mean	34.9	77.6	80.3	35.2	76.0	95.9	18.2	49.3	74.0	53.4	125.3	169.9	19.2	31.4	40.5
CV	20.5	10.4	11.9	23.7	9.7	10.5	31.2	16.0	14.5	21.3	9.4	9.6	15.2	13.3	9.7
LSD (0.05)	5.76	6.54	7.71	6.75	5.96	8.13	4.60	6.35	8.68	9.20	9.53	13.1	2.35	3.36	3.18

Table 4.9. Means of selected N related traits of 14 tropical maize hybrids at 0, 30 and 90 kg N/ha fertilizer application in Mokwa, Nigeria from 2006 to 2008.

HYBRIDS	Stover N content at silking (g/plant)			Grain N content (g/plant)			Total N content at maturity (g/plant)			Stover N accumulation (g/plant)			Total N accumulation (g/plant)		
	0	30	90	0	30	90	0	30	90	0	30	90	0	30	90
4001/4008	0.23	0.70	1.05	0.32	0.69	1.17	0.53	1.06	1.95	-0.02	-0.33	-0.26	0.30	0.36	0.91
KU 1409/4008	0.24	0.69	1.01	0.41	0.85	1.21	0.63	1.28	2.00	-0.03	-0.26	-0.22	0.39	0.59	0.99
9450/MOK Pion-Y-S4	0.23	0.79	1.24	0.20	0.60	1.20	0.33	0.93	1.84	-0.01	-0.45	-0.60	0.10	0.15	0.60
KU 1409/9613	0.27	0.84	1.19	0.32	0.65	1.21	0.47	0.98	1.89	-0.12	-0.51	-0.50	0.20	0.13	0.71
4008/1808	0.29	0.76	1.13	0.31	0.67	1.15	0.49	1.06	1.78	-0.11	-0.38	-0.50	0.20	0.29	0.65
4008/9071	0.38	0.67	1.26	0.28	0.59	0.88	0.50	0.98	1.50	-0.16	-0.28	-0.64	0.13	0.31	0.24
9613/9006	0.28	0.67	1.04	0.26	0.64	1.26	0.44	0.95	1.93	-0.10	-0.37	-0.37	0.16	0.26	0.89
4058/Fun 47-4	0.28	0.72	1.12	0.22	0.70	1.25	0.40	1.06	1.97	-0.10	-0.36	-0.40	0.13	0.34	0.85
1824/9432	0.24	0.78	0.97	0.27	0.58	1.12	0.46	0.86	1.70	-0.05	-0.50	-0.39	0.22	0.08	0.73
4058/GH 24	0.27	0.63	1.05	0.24	0.61	1.11	0.38	0.89	1.83	-0.13	-0.34	-0.34	0.11	0.27	0.78
9071/4058	0.31	0.76	1.11	0.23	0.66	1.22	0.38	0.99	1.87	-0.16	-0.43	-0.45	0.07	0.23	0.77
9006/4058	0.29	0.77	1.07	0.20	0.70	1.16	0.33	1.02	1.88	-0.17	-0.45	-0.36	0.03	0.25	0.80
OBA SUPER-1	0.31	0.67	1.08	0.17	0.65	1.03	0.39	0.98	1.72	-0.10	-0.34	-0.39	0.08	0.31	0.64
OBA SUPER-2	0.29	0.62	1.08	0.24	0.62	1.01	0.46	0.96	1.76	-0.09	-0.28	-0.33	0.15	0.34	0.68
Mean	0.28	0.72	1.10	0.26	0.66	1.14	0.44	1.00	1.83	-0.10	-0.38	-0.41	0.16	0.28	0.73
CV	21.4	16.0	15.1	29.6	16.4	15.3	19.8	12.9	12.0	-106.7	-32.0	-39.6	58.4	54.1	30.4
LSD (0.05)	0.05	0.09	0.13	0.06	0.09	0.14	0.07	0.10	0.18	0.06	0.10	0.13	0.08	0.17	0.26

Table 4.10. Analysis of variance of N-uptake efficiency at silking, N-uptake efficiency at maturity, N-utilization efficiency, N-use efficiency and Nitrogen harvest index at each of two N levels and for the combined N levels analysis in 14 tropical maize hybrids in Mokwa, Nigeria from 2006 to 2008.

N level	Source of variation	N-uptake efficiency at silking ($\times 10^{-1}$)	N-uptake efficiency at maturity ($\times 10^{-1}$)	N-utilization efficiency ($\times 10^{3}$)	N-use efficiency ($\times 10^{3}$)	Nitrogen harvest index ($\times 10^{-3}$)
low-N	Y	61.38***	34.83***	0.41ns	19.05***	13.09*
	H	2.67***	2.42***	2.91***	0.94***	6.00ns
	HxY	1.63***	2.42***	1.50***	1.80***	3.57ns
high-N	Y	35.61***	2.45***	15.22***	10.86***	540.06***
	H	0.20*	0.90***	0.26***	0.11***	8.13***
	HxY	0.56***	1.41***	0.41***	0.35***	9.87***
Combined N levels analysis	Y	94.10***	23.48***	9.56***	29.09***	351.06***
	N	70.96***	24.01***	27.04***	37.25***	104.60***
	H	1.65***	2.58***	2.06***	0.65***	9.58***
	YxN	2.90***	13.80***	6.08***	0.82***	202.09***
	HxY	1.65***	2.32***	1.33***	1.50***	7.19***
	HxN	1.22***	0.75*	1.12***	0.39***	4.55ns
	HxNxY	0.54**	1.50***	0.58***	0.65***	6.26**

*, **, ***: significant at 0.05, 0.01, 0.001 probability levels, respectively; ns: not significant; Y = year, N = N level, H = genotype.

Table 4.11. N-uptake efficiency, N-utilization efficiency, N-use efficiency and Nitrogen harvest index of 14 tropical maize hybrids at 30 (low-N) and 90 (high-N) kg N/ha in Mokwa, Nigeria from 2006 to 2008.

	N rate			
	low-N	high-N	LSD	
Traits	mean	mean	(0.05)	% reduction
At silking				
N-uptake efficiency	0.77	0.48	0.04	-60.4
At maturity				
Nitrogen harvest index	0.66	0.62	0.01	-6.5
N-uptake efficiency	0.99	0.82	0.04	-20.7
N-utilization efficiency	60.55	42.61	2.92	-42.1
N-use efficiency	55.36	34.30	2.09	-61.4

low-N, significant genotypic differences in NHI were observed only under high-N. The range in N-uptake efficiency for the hybrids at silking was 0.6 to 1.0 (mean = 0.77) at low-N and 0.4 to 0.6 (mean = 0.48) at high-N. N-uptake efficiency at maturity ranged from 0.7 to 1.2 (mean = 0.99) at low-N and 0.6 to 0.9 (mean =0.82) at high-N. N-utilization efficiency varied from 39.8 to 95.7 (mean = 60.55) at low-N and from 35.0 to 51.7 (mean = 42.61) at high-N. N-use efficiency ranged from 43.3 to 73.3 (mean = 55.36) at low-N and 29.8 to 38.3 (mean = 34.30) at high-N (Table 4.12). At silking, the six efficient and responsive hybrids (E1, E2, E4, E5, E8, and E9) had medium to high N-uptake efficiency under low-N. Three of these hybrids (E1, E4 and E9) showed below average N-uptake at maturity (Figures 4.2 and 4.3). The in-efficient and non-responsive hybrid, 9450/MOK Pion-Y-S4 (E3) had a high N-uptake at the two stages of sampling but a low N-utilization under both low-N and high-N. Among the efficient and responsive hybrids, E1, E4 and E9 had high N-utilization under both low-N and high-N while the remaining three had below average N-utilization under low-N (Figure 4.4). Although four of the high yielding hybrids under low-N (E1, E2, E4 and E5) had grain yields that did not differ significantly under high-N and low-N, they differed significantly in their N-uptake efficiency and N-utilization efficiency, particularly under low-N. While hybrids E2 and E5 had high N-uptake efficiency, hybrids E1 and E4 exhibited high N-utilization efficiency. The results show that in maize, both N-uptake efficiency and N-utilization efficiency contribute to high grain yield under high-N and low-N and hybrids could differ in the mechanism adopted to achieve N-use efficiency.

4.2 Inter-relationships among traits

4.2.1 Grain yield, yield components and agronomic traits

Grain yield was significantly ($p < 0.01$) correlated with number of kernels, one thousand kernel weight and kernel rows at both levels of N (Table 4.13). The correlation coefficient between one thousand kernel weight and grain yield at high-N was more than twice that at low-N. Among the yield components considered, number of kernels showed the highest level of association with grain yield at high-N and low-N. Kernel rows was related to number of kernels but not to one thousand kernel weight at high-N and low-N. Number of kernels was associated with other yield components at high-N but not correlated with one thousand kernel weight at low-N. Plant height was positively related ($p < 0.01$) to grain yield at both high-N and low-N. ASI was

Table 4.12. N-use efficiency and its components and nitrogen harvest index of 14 tropical maize hybrids at 30 (low-N) and 90 (high-N) kg N/ha fertilizer application in Mokwa, Nigeria from 2006 to 2008.

Hybrids	N-use efficiency		N-uptake efficiency at silking		N-uptake efficiency at maturity		N-utilization efficiency		Nitrogen harvest index	
	30	90	30	90	30	90	30	90	30	90
4001/4008	59.86	34.76	0.83	0.48	0.93	0.84	67.51	44.34	0.64	0.59
KU 1409/4008	46.53	29.78	0.78	0.45	1.16	0.81	44.66	37.31	0.66	0.61
9450/MOK Pion-Y-S4	60.33	35.53	0.94	0.59	1.07	0.89	53.90	35.22	0.64	0.63
KU 1409/9613	73.31	36.85	1.03	0.53	0.90	0.81	81.07	45.97	0.66	0.63
4008/1808	56.92	34.26	0.83	0.50	1.01	0.77	58.21	48.95	0.64	0.65
4008/9071	58.47	29.98	0.48	0.51	0.84	0.60	71.70	51.70	0.60	0.58
9613/9006	56.45	34.75	0.67	0.44	0.90	0.88	69.63	40.25	0.67	0.66
4058/Fun 47-4	56.37	35.61	0.77	0.49	1.16	0.93	49.64	38.63	0.66	0.63
1824/9432	68.76	37.23	0.92	0.42	0.72	0.74	95.66	47.27	0.67	0.65
4058/GH 24	54.85	34.91	0.68	0.48	0.91	0.86	62.48	39.92	0.68	0.61
9071/4058	46.56	38.34	0.77	0.46	1.08	0.88	42.69	43.75	0.67	0.64
9006/4058	46.91	37.81	0.87	0.47	1.22	0.92	39.79	40.80	0.68	0.60
OBA SUPER-1	46.43	30.12	0.63	0.46	1.05	0.79	52.24	41.86	0.66	0.61
OBA SUPER-2	43.31	30.29	0.60	0.47	0.88	0.77	58.50	40.51	0.65	0.58
Mean	55.36	34.30	0.77	0.48	0.99	0.82	60.55	42.61	0.66	0.62
CV	23.15	15.10	28.01	21.57	25.35	15.90	28.27	19.18	9.28	8.29
LSD (0.05)	10.36	4.19	0.17	0.08	0.20	0.11	13.83	6.60	0.05	0.04

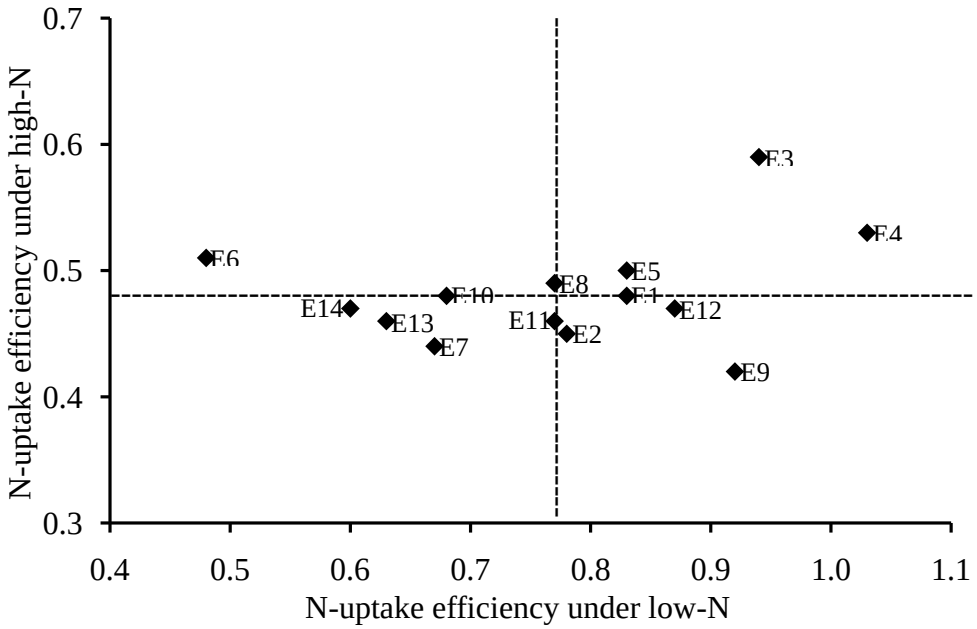

Figure 4.2. Biplot of N-uptake efficiencies at silking in 14 tropical maize hybrids at high and low levels of applied nitrogen fertilizer. Broken lines represent mean N-uptake efficiencies.

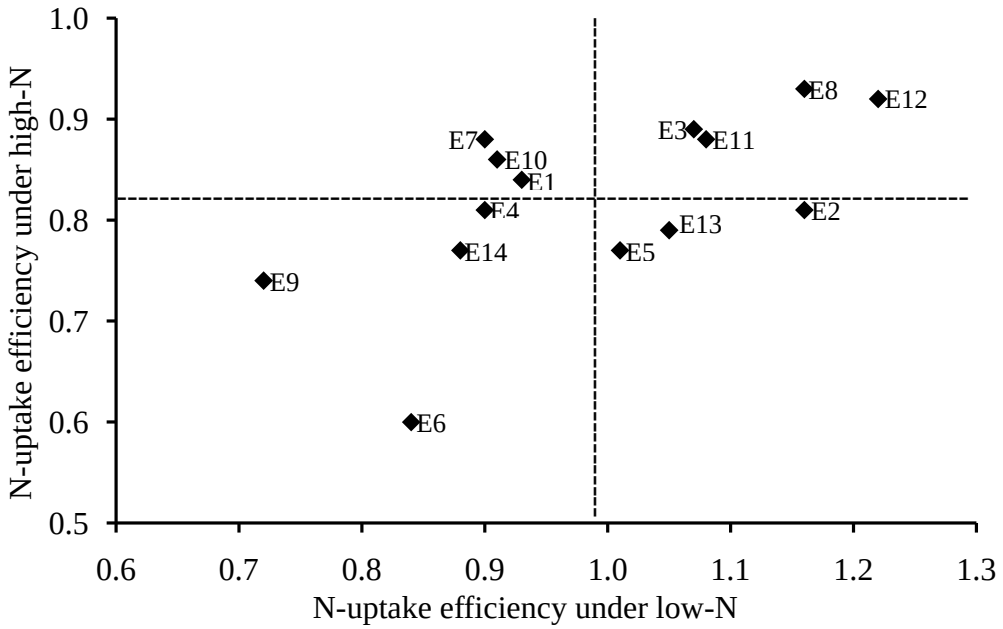

Figure 4.3. Biplot of N-uptake efficiencies at maturity in 14 tropical maize hybrids at high and low levels of applied nitrogen fertilizer. Broken lines represent mean N-uptake efficiencies.

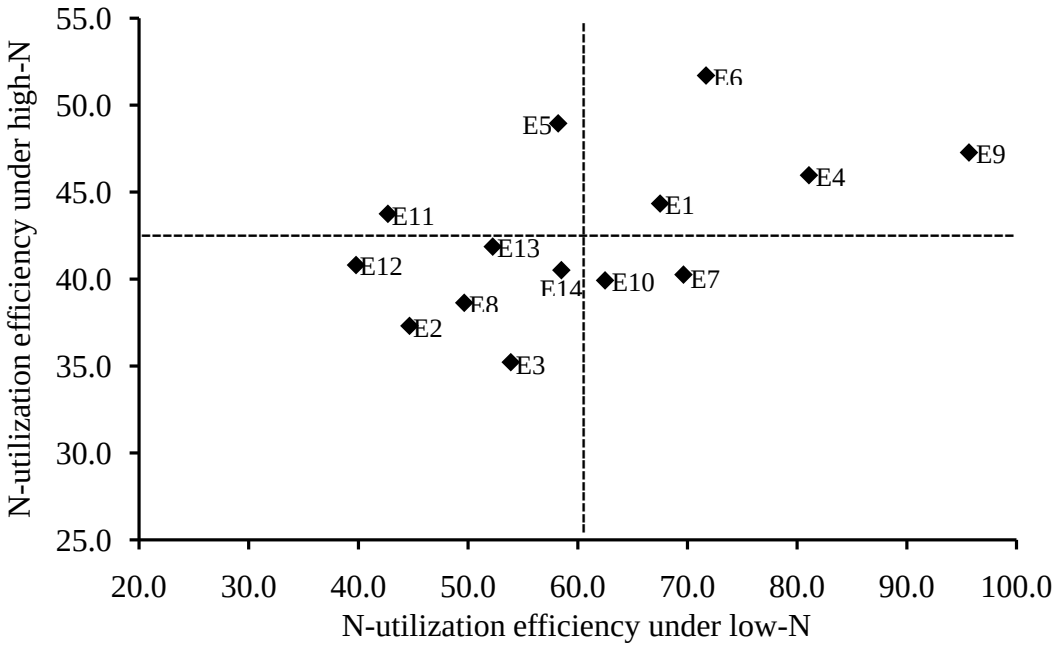

Figure 4.4. Biplot for N-utilization efficiencies in 14 tropical maize hybrids at high and low levels of applied nitrogen fertilizer. Broken lines represent mean N-utilization efficiencies.

Table 4.13. Correlation coefficients among selected agronomic and yield traits of 14 tropical maize hybrids at 30 (low-N, above the diagonal)) and 90 (high-N, below the diagonal) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	Plant height (m)	Anthesis-silking interval (days)	Kernel rows	Number of kernels	One thousand kernel weight (g)	Grain yield (Mg/ha^{-1})
Plant height (m)		-0.32**	0.47**	0.61**	0.10ns	0.66**
Anthesis – silking interval (days)	-0.15*		-0.27**	-0.33**	0.11ns	-0.28**
Kernel rows	0.30**	-0.02ns		0.48**	-0.06ns	0.36**
Number of kernels	0.80**	-0.09ns	0.45**		0.13ns	0.79**
One thousand kernel weight (g)	0.49**	-0.04ns	0.02ns	0.52**		0.26**
Grain yield (Mg/ha)	0.82**	-0.15ns	0.25**	0.81**	0.62**	

*, **: significant at 0.05, 0.01 probability levels, respectively; ns: not significant.

negatively related ($p < 0.01$) to grain yield and its components, except one thousand kernel weight, but the associations were significant only at low-N.

4.2.2 Grain yield and physiological traits

The correlations of grain yield and number of kernels with ear leaf chlorophyll content (SPAD) were high and positive ($p < 0.01$) at high-N and low-N. Grain yield was positively related ($p < 0.01$) to plant biomass recorded at silking and maturity under low-N and high-N. Grain yield showed significant ($p < 0.01$) association with post-silking stover and total dry matter accumulation at high-N, but its correlation was only significant ($p < 0.01$) with post-silking total dry matter accumulation under low-N. The relationships between number of kernels and these traits followed a similar trend as grain yield (Table 4.14).

A significant ($p < 0.01$) positive correlation was observed between grain yield and stover N concentration at silking under high-N and low-N. However, the relationship between grain yield and stover N concentration at maturity was negative, but with the correlation coefficient at high-N being more than thrice that at low-N. The relationship between grain yield and stover N content at silking was positive at high-N and low-N. At maturity, the association between grain yield and stover N content was positive at low-N but was negative under high-N. The relationship between grain yield and grain N concentration was positive but low at low-N, and negative at high-N. The association between grain yield and Grain N content was positive and similar at high-N and low-N. N accumulation (stover and total) between silking and maturity was negatively correlated with grain yield at high-N and low-N. Of the yield components, number of kernels showed the highest positive association with stover N content at silking under high-N and low-N (Table 4.15).

Stover N content at silking exhibited high positive relationship with plant dry matter both at silking and maturity at high-N and low-N. At maturity, stover N content showed a positive correlation with stover dry matter at silking and maturity under low-N condition, while the relationships were negative and relatively low at high-N. Stover N accumulation from silking to maturity was negatively related to dry matter production both at silking and maturity and at high-N and low-N (Table 4.16).

4.2.3 Grain yield and efficiencies of N-use, N-uptake and N-utilization

Grain yield showed positive and significant association with N-use, N-utilization and N-uptake efficiency both at silking and maturity. However, the

Table 4.14. Correlation coefficients between selected agronomic and yield traits and physiological traits of 14 tropical maize hybrids grown under 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	N level	Plant height (m)	Anthesis-silking interval (days)	Kernel rows	Number of kernels	One thousand kernel weight (g)	Grain yield (Mg/ha)
Stover dry weight at silking (g/plant)	low-N	0.63**	-0.26**	0.40**	0.68**	0.23**	0.78**
	high-N	0.76**	-0.16**	0.23**	0.72**	0.54**	0.80**
Ear leaf chlorophyll (SPAD)	low-N	0.57**	-0.17*	0.28**	0.68**	0.35**	0.75**
	high-N	0.74**	-0.14ns	0.16*	0.76**	0.63**	0.85**
Stover dry weight at maturity (g/plant)	low-N	0.62**	-0.28**	0.21**	0.68**	0.25**	0.75**
	high-N	0.79**	-0.15*	0.27**	0.75**	0.55**	0.84**
Total dry weight (g/plant)	low-N	0.63**	-0.26**	0.26**	0.68**	0.26**	0.74**
	high-N	0.80**	-0.13ns	0.31**	0.77**	0.57**	0.86**
Stover dry matter accumulation (g/plant)	low-N	-0.02ns	-0.02ns	-0.24**	-0.00ns	0.03ns	-0.03ns
	high-N	0.43**	-0.06ns	0.19*	0.40**	0.27**	0.47**
Total dry matter accumulation (g/plant)	low-N	0.29**	-0.13ns	-0.01ns	0.33**	0.16*	0.31**
	high-N	0.70**	-0.09ns	0.31**	0.69**	0.51**	0.76**

*, **: significant at 0.05, 0.01 probability levels, respectively; ns: not significant.

Table 4.15. Correlation coefficients between selected agronomic and yield traits and N related traits in 14 tropical maize hybrids grown under 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	N level	Plant height (m)	Anthesis-silking interval (days)	Kernel rows	Number of kernels	One thousand kernel weight (g)	Grain yield (Mg/ha)
Stover N concentration at silking (g/plant)	low-N	0.46**	-0.38**	0.15ns	0.52**	-0.12ns	0.57**
	high-N	0.36**	-0.23**	0.02ns	0.20**	-0.05ns	0.35**
Stover N content at silking (g/plant)	low-N	0.63**	-0.35**	0.31**	0.68**	0.07ns	0.77**
	high-N	0.75**	-0.24**	0.18*	0.63**	0.38**	0.77**
Grain N concentration (%)	low-N	0.22**	-0.31**	-0.09ns	0.18*	-0.16*	0.24**
	high-N	-0.50**	-0.15*	-0.24**	-0.57**	-0.51**	-0.49**
Grain N content (g/plant)	low-N	0.55**	-0.29**	0.23**	0.57**	0.16*	0.60**
	high-N	0.62**	-0.19*	0.23**	0.56**	0.42**	0.68**
Stover N concentration at maturity (g/plant)	low-N	-0.35**	-0.09ns	-0.27**	-0.20**	-0.18*	-0.24**
	high-N	-0.70**	-0.05ns	-0.16*	-0.74**	-0.57**	-0.73**
Stover N content at maturity (g/plant)	low-N	0.28**	-0.15*	-0.02ns	0.43**	0.13ns	0.47**
	high-N	-0.32**	-0.20*	0.04ns	-0.37**	-0.23**	-0.34**
Total N content (g/plant)	low-N	0.52**	-0.28**	0.16*	0.60**	0.17*	0.63**
	high-N	0.34**	-0.28**	0.21**	0.26**	0.22**	0.38**
Stover N accumulation (g/plant)	low-N	-0.59**	0.34**	-0.35**	-0.60**	-0.03ns	-0.68**
	high-N	-0.77**	0.13ns	-0.14ns	-0.69**	-0.41**	-0.80**
Total N accumulation (g/plant)	low-N	-0.26**	0.17*	-0.22**	-0.26**	0.08ns	-0.33**
	high-N	-0.51**	0.02ns	-0.01ns	-0.45**	-0.21**	-0.50**

*, **: significant at 0.05, 0.01 probability levels, respectively; ns: not significant.

Table 4.16. Correlation coefficients between physiological traits and N related traits in 14 tropical maize hybrids grown under 30 (low-N) and 90 (high-N) kg N ha^{-1} fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	N level	Stover weight at silking (g/plant)	Ear leaf chlorophyll (SPAD)	Stover weight at maturity (g/plant)	Total above-ground dry weight (g/plant)	Total dry matter accumulation (g/plant)
Stover N concentration at silking (g/plant)	low-N	0.58**	0.41**	0.51**	0.48**	0.14ns
	high-N	0.24**	0.32**	0.29**	0.31**	0.31**
Stover N content at silking (g/plant)	low-N	0.89**	0.62**	0.67**	0.65**	0.09ns
	high-N	0.87**	0.72**	0.84**	0.81**	0.64**
Grain N concentration (%)	low-N	0.25**	0.11ns	0.22**	0.17*	0.02ns
	high-N	-0.56**	-0.43**	-0.52**	-0.54**	-0.44**
Grain N content (g/plant)	low-N	0.57**	0.50**	0.69**	0.86**	0.65**
	high-N	0.59**	0.64**	0.70**	0.82**	0.87**
Stover N concentration at maturity (g/plant)	low-N	-0.34**	-0.23**	-0.25**	-0.26**	-0.05ns
	high-N	-0.76**	-0.70**	-0.74**	-0.75**	-0.62**
Stover N content at maturity (g/plant)	low-N	0.32**	0.40**	0.67**	0.61**	0.54**
	high-N	-0.25**	-0.36**	-0.19*	-0.24**	-0.19*
Total N content (g/plant)	low-N	0.55**	0.53**	0.77**	0.88**	0.70**
	high-N	0.35**	0.33**	0.48**	0.56**	0.62**
Stover N accumulation (g/plant)	low-N	-0.87**	-0.54**	-0.50**	-0.50**	0.08ns
	high-N	-0.85**	-0.76**	-0.79**	-0.80**	-0.63**

*, **: significant at 0.05, 0.01probability levels, respectively; ns: not significant.

correlation coefficient between grain yield and N-utilization efficiency at high-N was more than twice its corresponding value at low-N. N-utilization efficiency also had higher correlation with number of kernels at high-N than at low-N (Table 4.17).

4.2.4 N-use efficiency and its components

N-use efficiency showed significant ($p < 0.01$) positive correlation with N-utilization efficiency at both levels of N, with a stronger association at high-N (0.79) than at low-N (0.51). A similar trend was also observed between N-use efficiency and N-uptake efficiency at silking (Table 4.18). At maturity, the correlation coefficient between N-use efficiency and N-uptake efficiency was positive and similar at high-N and low-N. The correlation between N-use efficiency and NHI was high and significant ($p < 0.01$) at high-N, but was very low and non-significant at low-N. N-uptake efficiency at silking was related ($p < 0.01$) to N-utilization efficiency only at high-N. At maturity, N-uptake efficiency and N-utilization efficiency were negatively related at both levels of N, with the correlation coefficient at low-N being more than twice that at high-N.

4.2.5 N-use efficiency and physiological traits

N-uptake efficiency at silking exhibited high positive correlation ($p < 0.01$) with stover dry weight both at silking and maturity under high-N and low-N. The association of N-uptake efficiency at maturity with stover dry weight at silking and maturity were significant ($p < 0.01$) and similar under high-N and low-N. N-utilization efficiency showed positive and significant ($p < 0.01$) association with stover dry matter at silking, with the correlation coefficient at high-N being more than twice that at low-N. At maturity, N-utilization was significantly ($p < 0.01$) correlated with stover dry weight only at high-N. Stover dry matter accumulation from silking to maturity was negatively related to N-utilization under low-N. The relationship between N-use efficiency and dry matter production was positive ($p < 0.01$) irrespective of the N and the stage of growth. The relationships between ear leaf chlorophyll content (SPAD) on the one hand, and N-use efficiency and its components on the other, were positive and significant ($p < 0.01$) (Table 4.19).

At silking, N-uptake efficiency had high positive correlations with stover N content at silking at both high-N and low-N. Stover N content at silking showed positive and significant ($p < 0.01$) association with N-use efficiency and its components with higher correlation coefficients at high-N relative to low-N. Grain N

Table 4.17. Correlation coefficients between agronomic, yield and N related traits in 14 tropical maize hybrids grown under 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	N level	Plant height (m)	Anthesis-silking interval (days)	Kernel rows	Number of kernels	One thousand kernel weight (g)	Grain yield (Mg/ha)
N-uptake efficiency at silking	low-N	0.57**	-0.23**	0.33**	0.64**	0.21**	0.68**
	high-N	0.75**	-0.16*	0.19*	0.65**	0.44**	0.77**
N-uptake efficiency at maturity	low-N	0.36**	-0.05ns	0.13ns	0.38**	0.33**	0.41**
	high-N	0.33**	-0.14ns	0.24**	0.28**	0.26**	0.36**
N-utilization efficiency	low-N	0.15*	-0.10ns	0.22**	0.23**	0.05ns	0.33**
	high-N	0.59**	-0.01ns	0.15ns	0.67**	0.51**	0.72**
N-use efficiency	low-N	0.50**	-0.19*	0.32**	0.62**	0.35**	0.76**
	high-N	0.74**	-0.04ns	0.27**	0.77**	0.60**	0.90**
Nitrogen harvest index	low-N	0.29**	-0.15*	0.24**	0.15ns	0.05ns	0.14ns
	high-N	0.62**	-0.01ns	0.12ns	0.63**	0.45**	0.67**

*, **: significant at 0.05, 0.01probability levels, respectively; ns: not significant.

Table 4.18. Correlation coefficients for N-use efficiency traits in 14 tropical maize hybrids grown under 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008 (low-N above and high-N below the diagonal).

	N-uptake efficiency at silking	N-uptake efficiency at maturity	N-utilization efficiency	N-use efficiency	Nitrogen harvest index
N-uptake efficiency at silking		0.44**	0.12ns	0.57**	0.21**
N-uptake efficiency at maturity	0.43**		-0.45**	0.45**	0.20**
N-utilization efficiency	0.44**	-0.17*		0.51**	-0.04ns
N-use efficiency	0.67**	0.42**	0.79**		0.12ns
Nitrogen harvest index	0.55**	0.17*	0.56**	0.63**	

*, **: significant at 0.05, 0.01 probability levels, respectively; ns: not significant.

Table 4.19. Correlation coefficients between selected agronomic traits and N-use efficiency traits in 14 tropical maize hybrid grown under 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	N level	N-uptake efficiency at silking	N-uptake efficiency at maturity	N-utilization efficiency	N-use efficiency	Nitrogen harvest index
Stover dry weight at silking (g/plant)	low-N	0.84**	0.37**	0.23**	0.59**	0.25**
	high-N	0.90**	0.39**	0.56**	0.73**	0.53**
Ear leaf chlorophyll content (SPAD)	low-N	0.62**	0.38**	0.23**	0.59**	0.08ns
	high-N	0.72**	0.29**	0.62**	0.77**	0.65**
Stover dry weight at maturity (g/plant)	low-N	0.57**	0.51**	-0.05ns	0.42**	0.03ns
	high-N	0.86**	0.50**	0.48**	0.74**	0.55**
Total dry weight (g/plant)	low-N	0.59**	0.64**	-0.16*	0.42**	0.25**
	high-N	0.86**	0.57**	0.47**	0.77**	0.68**
Stover dry matter accumulation (g/plant)	low-N	-0.33**	0.17*	-0.35**	-0.22**	-0.27**
	high-N	0.33**	0.45**	0.05ns	0.36**	0.29**
Total dry matter accumulation (g/plant)	low-N	0.05ns	0.53**	-0.42**	0.04ns	0.11ns
	high-N	0.66**	0.62**	0.31**	0.68**	0.68**

*, **: significant at 0.05, 0.01probability levels, respectively; ns: not significant.

content was significantly ($p < 0.01$) and positively correlated with N-uptake efficiency both at silking and maturity, N-use efficiency and NHI, with the correlation efficient being higher at high-N compared with low-N. The relationship of Grain N content with N-utilization was negative at low-N and positive at high-N. Stover N accumulation from silking to maturity was negatively correlated with N-use efficiency and its components as well as NHI (Table 4.20).

4.3 Determining the relative contribution of selected traits to N-use, number of kernels and grain yield

4.3.1 Path analysis of N-uptake efficiency and N-utilization efficiency to N-use efficiency

The direct and indirect causes of association of the two main components of N-use efficiency (namely N-uptake and N-utilization efficiency) at both levels of N, obtained from path analysis are presented in Table 4.21. The direct and indirect effects of these two traits explained about 76% and 63% of the variation observed for N-use efficiency under high-N and low-N conditions, respectively. At both stages of sampling, the direct effect of N-uptake efficiency on N-use efficiency under low-N was found to be higher than under high-N. This is suggestive of a relative higher importance of N-uptake under N-limiting conditions. The direct effect of N-utilization efficiency on N-use efficiency under both levels of N was high and comparable. However, the higher negative indirect effect through N-uptake efficiency at maturity under low-N resulted in a lower correlation. This suggests that N-utilization efficiency is an important component of N-use efficiency at both N levels. The indirect effects of N-utilization efficiency and N-uptake efficiency at maturity on N-use efficiency, coupled with the negative associations between the two components of N-use efficiency both at high-N and low-N, suggests that it may be difficult to improve these two traits simultaneously.

4.3.2 Path analysis of different traits to number of kernels

Given the result earlier presented in Table 4.14, showing number of kernels as the most important grain yield component at high-N and low-N, path coefficient analysis showed that N-use efficiency, N-uptake efficiency, N-utilization efficiency, kernel rows, ear leaf chlorophyll content (SPAD), and stover N content at silking accounted for 52% and 42% of the variation observed in number of kernels under high-N and low-N respectively (Table 4.22). While the positive direct effect of N-

Table 4.20. Correlation coefficients between selected N related traits and N-use efficiency traits in 14 tropical maize hybrids grown under 30 (low-N) and 90 (high-N) kg N/ha fertilizer application at Mokwa, Nigeria from 2006 to 2008.

	N level	N-uptake efficiency at silking	N-uptake efficiency at maturity	N-utilization efficiency	N-use efficiency	Nitrogen harvest index
Stover N concentration at silking (g/plant)	low-N	0.71**	0.14ns	0.03ns	0.24**	0.07ns
	high-N	0.57**	0.18*	-0.02ns	0.11ns	0.23**
Stover N content at silking (g/plant)	low-N	0.89**	0.31**	0.15*	0.50**	0.18*
	high-N	0.97**	0.41**	0.40**	0.61**	0.52**
Grain N concentration (%)	low-N	0.23**	0.02ns	-0.09ns	-0.02ns	0.07ns
	high-N	-0.40**	0.05ns	-0.65**	-0.56**	-0.44**
Grain N content (g/plant)	low-N	0.52**	0.63**	-0.28**	0.30**	0.46**
	high-N	0.67**	0.75**	0.15*	0.59**	0.66**
Stover N concentration at maturity (g/plant)	low-N	-0.21**	-0.04ns	-0.26**	-0.27**	-0.66**
	high-N	-0.66**	-0.05ns	-0.74**	-0.72**	-0.82**
Stover N content at maturity (g/plant)	low-N	0.32**	0.42**	-0.22**	0.18*	-0.49**
	high-N	-0.21**	0.41**	-0.62**	-0.38**	-0.75**
Total N content (g/plant)	low-N	0.52**	0.64**	-0.29**	0.29**	0.17*
	high-N	0.45**	0.87**	-0.23**	0.28**	0.12ns
Stover N accumulation (g/plant)	low-N	-0.86**	-0.20**	-0.24**	-0.49**	-0.37**
	high-N	-0.91**	-0.19*	-0.59**	-0.67**	-0.75**

*, **: significant at 0.05, 0.01probability levels, respectively; ns: not significant.

Table 4.21. Direct (in bold on the diagonal) and indirect effects of N-uptake efficiency and N-utilization efficiency on N-use efficiency in 14 tropical maize hybrids at 30 (low-N) and 90 (high-N) kg N/ha levels of nitrogen fertilizer.

	Traits	N Level	Indirect effects via 1	2	3	Total correlation with N-use efficiency
1	N-uptake efficiency at silking	high-N	**0.042**	0.241	0.384	0.667
		low-N	**0.143**	0.328	0.098	0.569
2	N-uptake efficiency at maturity	high-N	0.018	**0.544**	-0.145	0.417
		low-N	0.061	**0.770**	-0.379	0.452
3	N-utilization efficiency	high-N	0.018	-0.091	**0.867**	0.795
		low-N	0.017	-0.346	**0.842**	0.512

Table 4.22. Direct (in bold on the diagonal) and indirect effects of selected agro-physiological traits on number of kernels in 14 tropical maize hybrids at 30 (low-N) and 90 (high-N) kg N/ha levels of nitrogen fertilizer.

			Indirect effect via						Total correlation with number of kernels
Trait		N level	1	2	3	4	5	6	
1	N-uptake efficiency at silking	high-N	**0.143**	0.044	0.156	0.055	0.273	-0.018	0.653
		low-N	**-0.162**	0.100	0.019	0.080	0.189	0.414	0.640
2	N-uptake efficiency at maturity	high-N	0.064	**0.099**	-0.059	0.072	0.112	-0.008	0.280
		low-N	-0.069	**0.235**	-0.074	0.031	0.114	0.146	0.384
3	N-utilization efficiency	high-N	0.064	-0.017	**0.352**	0.044	0.234	-0.007	0.670
		low-N	-0.019	-0.106	**0.164**	0.052	0.069	0.071	0.231
4	Kernel rows	high-N	0.027	0.024	0.053	**0.295**	0.059	-0.003	0.455
		low-N	-0.054	0.031	0.036	**0.236**	0.086	0.144	0.479
5	Chlorophyll content (SPAD)	high-N	0.103	0.029	0.217	0.046	**0.380**	-0.013	0.762
		low-N	-0.101	0.088	0.037	0.067	**0.304**	0.288	0.684
6	Stover N content at silking	high-N	0.139	0.040	0.141	0.053	0.273	**-0.018**	0.627
		low-N	-0.144	0.074	0.025	0.073	0.188	**0.466**	0.682

uptake efficiency at maturity on number of kernels under low-N was more than two-fold that under high-N, the reverse was the case for N-utilization efficiency for which the direct effect on number of kernels at high-N was twice that under low-N. Thus, N-uptake efficiency was more important for number of kernels and grain yield under low-N, while N-utilization efficiency was more important for number of kernels and grain yield under high-N. These results indicate a difference in the relative importance of N-use components on number of kernels under the two N levels. The positive correlations between the traits included in the path analysis with number of kernels under low-N condition could be attributed to the direct effect of stover N content at silking on number of kernels.

4.3.3 Path analysis of selected traits to grain yield

Result of path coefficient analysis of the effects of nine selected traits on grain yield is presented in Table 4.23. These traits were able to explain 73% and 63% of the variation observed in grain yield under high-N and low-N conditions, respectively. The analysis revealed that stover N content at silking followed by N-use efficiency had the most direct effect on grain yield at both levels of N application. The direction of the effects of both traits on grain yield suggests that both traits could be simultaneously improved to improve grain yield. As expected, the importance of chlorophyll content to yield was more pronounced under low-N conditions, with the direct effect being more than two-folds under low-N that what was obtained under high-N. The two components of N-use efficiency showed negative paths on grain yield at both N levels. The positive correlations of these traits on grain yield seem to be essentially a consequence of the indirect effects both N-use efficiency and stover N content at silking have on these traits. It is noteworthy however that although these modulatory roles resulted in comparable correlations between N-uptake efficiency and grain yield at both low-N and high-N, the same could not be said of N-utilization efficiency where the indirect effects of the two traits was lower at low-N relative to high-N. This resulted in the correlation coefficient between N-utilization efficiency and grain yield at high-N being more than twice that low-N. This could be a further reflection of the greater importance of N-utilization efficiency under high-N application. Of the grain yield components included in the path analysis, number of kernels was the most important trait, particularly under sub-optimal N condition.

Table 4.23. Direct (in bold on the diagonal) and indirect effects of selected agro-physiological traits on grain yield of 14 tropical maize hybrids at 30 (low-N) and 90 (high-N) kg N/ha levels of nitrogen fertilizer.

Traits		N level	Indirect effect via									Total correlation with grain yield
			1	2	3	4	5	6	7	8	9	
1	N-use efficiency	high-N	**0.703**	-0.333	-0.052	-0.097	0.081	0.072	0.003	0.067	0.457	0.899
		low-N	**0.437**	-0.223	-0.013	-0.031	0.121	0.022	0.007	0.129	0.326	0.764
2	N-uptake efficiency at silking	high-N	0.468	**-0.500**	-0.055	-0.054	0.068	0.053	0.002	0.063	0.726	0.772
		low-N	0.248	**-0.392**	-0.012	-0.007	0.125	0.013	0.007	0.125	0.577	0.684
3	N-uptake efficiency at maturity	high-N	0.293	-0.222	**-0.123**	0.020	0.029	0.031	0.002	0.026	0.307	0.364
		low-N	0.197	-0.167	**-0.029**	0.028	0.075	0.020	0.003	0.076	0.204	0.407
4	N-utilization efficiency	high-N	0.558	-0.221	0.021	**-0.122**	0.070	0.061	0.001	0.054	0.302	0.724
		low-N	0.224	-0.046	0.013	**-0.061**	0.045	0.003	0.004	0.046	0.099	0.328
5	Number of kernels	high-N	0.543	-0.327	-0.035	-0.082	**0.104**	0.062	0.004	0.067	0.471	0.809
		low-N	0.271	-0.251	-0.011	-0.014	**0.195**	0.008	0.010	0.138	0.443	0.790
6	Kernel weight	high-N	0.419	-0.222	-0.032	-0.062	0.054	**0.120**	0.000	0.055	0.284	0.617
		low-N	0.153	-0.084	-0.009	-0.003	0.027	**0.062**	-0.001	0.070	0.045	0.259
7	Kernel rows	high-N	0.189	-0.094	-0.030	-0.018	0.048	0.002	**0.009**	0.014	0.135	0.254
		low-N	0.141	-0.132	-0.004	-0.013	0.094	-0.003	**0.020**	0.057	0.201	0.360
8	Chlorophyll content (SPAD) at silking	high-N	0.538	-0.359	-0.036	-0.075	0.080	0.076	0.001	**0.088**	0.539	0.851
		low-N	0.258	-0.244	-0.011	-0.014	0.134	0.021	0.006	**0.202**	0.401	0.754
9	Stover N content at silking	high-N	0.427	-0.484	-0.050	-0.049	0.066	0.045	0.002	0.063	**0.751**	0.771
		low-N	0.219	-0.349	-0.009	-0.009	0.133	0.004	0.006	0.125	**0.649**	0.770

4.4 Microarray results

4.4.1 Differentially expressed gene

Samples of immature ears from four tropical maize hybrids, namely KU1409/9613 (E4), KU1409/4008 (E2), 4001/4008 (E1) and Oba Super-1 (E13) grown under low-N were collected at anthesis. These hybrids were selected for the microarray study based on their performances in the field. While E4, E2 and E1 were the highest yielding hybrids under low-N and were among the top eight high yielding hybrids under high-N, E13 was the most inefficient and non-responsive, and the lowest yielding hybrid under both high-N and low-N. Using the Maize Oligonucleotide arrays as probes and the commercial N inefficient and non-responsive hybrid (E13) as a reference, the samples were subjected to microarray analysis. Out of the ~57,000 tested probes on the Maize Oligonucleotide arrays, the number of probes differentially expressed was 2132 for E4, 3585 for E2, and 2117 for E1. Among the probes showing differential expression, 1144 were differentially expressed in more than one hybrid. A total of 163 (~ 0.3%) genes were differentially expressed across the three hybrids (Figure 4.5).

Both up- and down-regulation of genes occurred in the three hybrids. About 74% of the differentially expressed genes in E4 were down regulated while 58% and 47% of the differentially expressed genes were down regulated in E2 and E1, respectively (Figure 4.6). About 30% of the 163 differentially expressed genes common to the three hybrids were either similar to proteins of unknown functions or they lacked functional annotation. Among the 163 differentially expressed genes common to the three hybrids, only two (L-asparaginase and glutamine synthetase) were known to be directly involved in the asparagine (Asn) cycling pathway. While glutamine synthetase was consistently up-regulated in all three hybrids, L-asparaginase (ASNase) was up-regulated in E4 and E2 but down regulated in E1 (Table 4.24). Information about the 163 differentially expressed genes is presented in Appendix 3.

4.4.2 Microarray data validation

To evaluate the validity of gene expression analysis using microarrays, real-time qRT-PCR analysis was conducted on 9 genes that showed expression differences in the three hybrids (Table 4.24). The result showed some level of consistency in terms of direction between the microarray analysis and the real-time qPCR data with respect

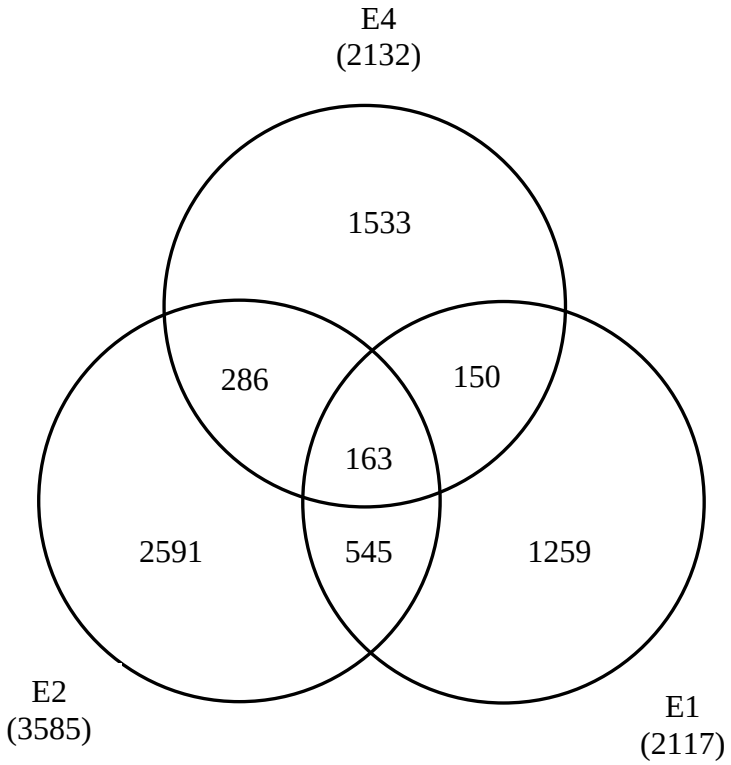

Figure 4.5. Number of genes differentially expressed in immature maize earshoots harvested at silking in the three hybrids (E1, E2, and E4) relative to the reference hybrid (E13).

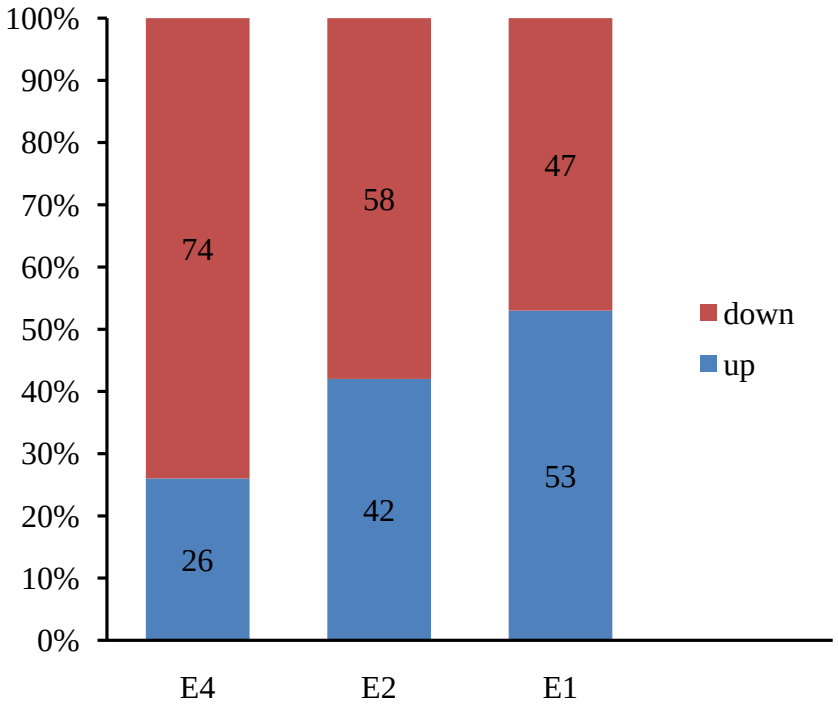

Figure 4.6. Percentage of up- and down- regulated genes in the three hybrids (E4, E2 and E1) relative to the reference hybrid (E13).

Table 4.24. Genes used for data validation by qPCR. The gene transcripts are presented relative to that of the reference hybrid (E13).

ID	Putative annotation	Fold change					
		qRT-PCR			Microarray		
		E4	E2	E1	E4	E2	E1
MZ00004043	Sucrose synthase 1 (EC 2.4.1.13)	1.02	1.56	1.85	1.00	1.00	2.16
MZ00017849	Ras-related protein rab11d	1.48	1.75	3.17	3.22	2.29	2.50
MZ00019244	L-asparaginase 2	13.91	9.69	1.47	2.91	2.90	0.46
MZ00021642	Putative bZIP transcription factor RF2b	2.42	2.34	1.27	3.28	1.00	1.00
MZ00024296	1-aminocycloprotein-1-carboxylase oxidase	1.06	1.57	0.57	3.08	1.00	2.99
MZ00025484	14-3-3-like protein	1.55	1.53	1.57	4.80	9.98	9.57
MZ00027609	Glutamine synthetase	2.67	2.59	3.24	2.14	4.29	2.42
MZ00042156	Homeobox protein rough sheath 1	1.04	2.17	0.91	9.06	8.94	3.57
MZ00042579	bHLH transcription factor PTF1 {Oryza sativa;}	0.74	1.36	1.74	2.36	1.71	3.03

to the relative direction of expression. This was particularly clear for E1 and E4.

4.4.3 Expression of L-asparaginase in developing ear shoots of maize

The major metabolites of the Asparagine cycling pathway (asparagine, glutamine, glutamate and aspartate) make important contributions to N transport, connecting N metabolism, photosynthesis and C/N balance in higher plants. Of the key genes connected with this pathway, only L-asparaginase (MZ00019244) and glutamine synthetase(MZ00027609) were the differentially expressed genes common to the three hybrids. Because of the positive correlation (r=0.95*) between the results of the microarray and qRT-PCR analyses for L-asparaginase (ASNase) gene expression and its importance as a key factor in the asparagine cycling pathway, its qPCR expression profiling was extended to all 14 maize hybrids used in the study. The result of this analysis (Table 4.25; Figure 4.7) is presented as fold-change normalized to GAPDH and relative to the expression observed in the highest yielding hybrid under low-N KU1409/9613 (E4). Results from this analysis revealed that ASNase gene expression was consistently down regulated relative to E4. Furthermore, ASNase gene expression showed a positive and significant correlation with grain yield, number of kernels and N-utilization efficiency. The correlation of ASNase gene with N-use efficiency was only significant at 0.10 level of probability and was not significant with N-uptake efficiency both at silking and maturity (Table 4.26).

Table 4.25. Relative quantitation of L-asparaginase (ASNase) gene expression in 14 tropical maize hybrids with GAPDH as housekeeping gene and expression relative to the hybrid KU1409/9613.

Genotype	ASNase Average C_T	GAPDH Average C_T	ΔC_T	$\Delta\Delta C_T$	Relative expression
KU1409/9613	25.41±0.26	22.87±0.06	2.54±0.26	0.00±0.26	1.00 (0.83 – 1.20)
KU1409/4008	25.10±0.10	22.07±0.07	3.03±0.12	0.49±0.12	0.71 (0.65 – 0.77)
4058/Fun.47-3	26.39±0.29	22.59±0.03	3.80±0.29	1.26±0.29	0.42 (0.34 – 0.51)
4008/1808	27.27±0.13	23.37±0.39	3.90±0.41	1.37±0.41	0.39 (0.29 – 0.52)
OBA SUPER-2	26.11±0.08	22.12±0.27	3.99±0.28	1.45±0.28	0.37 (0.30 – 0.44)
9450/MOK Pion-Y-S4	26.28±0.24	22.20±0.19	4.07±0.30	1.54±0.30	0.34 (0.28 – 0.42)
4008/9071	26.69±0.21	22.62±0.17	4.08±0.27	1.54±0.27	0.34 (0.29 – 0.41)
9006/4058	26.76±0.33	22.33±0.25	4.43±0.42	1.90±0.42	0.27 (0.20 – 0.36)
1824/9432	27.77±0.05	23.17±0.15	4.60±0.16	2.06±0.16	0.24 (0.21 – 0.27)
4058/GH24	27.77±0.06	23.12±0.05	4.65±0.08	2.12±0.08	0.23 (0.22 – 0.24)
9613/9006	27.11±0.23	22.39±0.17	4.72±0.28	2.18±0.28	0.22 (0.18 – 0.27)
OBA SUPER-1	27.87±0.38	22.49±0.47	5.38±0.60	2.84±0.60	0.14 (0.09 – 0.21)
4001/4008	28.65±0.48	22.94±0.07	5.71±0.49	3.17±0.49	0.11 (0.08 – 0.16)
9071/4058	27.78±0.14	22.06±0.07	5.72±0.16	3.19±0.16	0.11 (0.10 – 0.12)

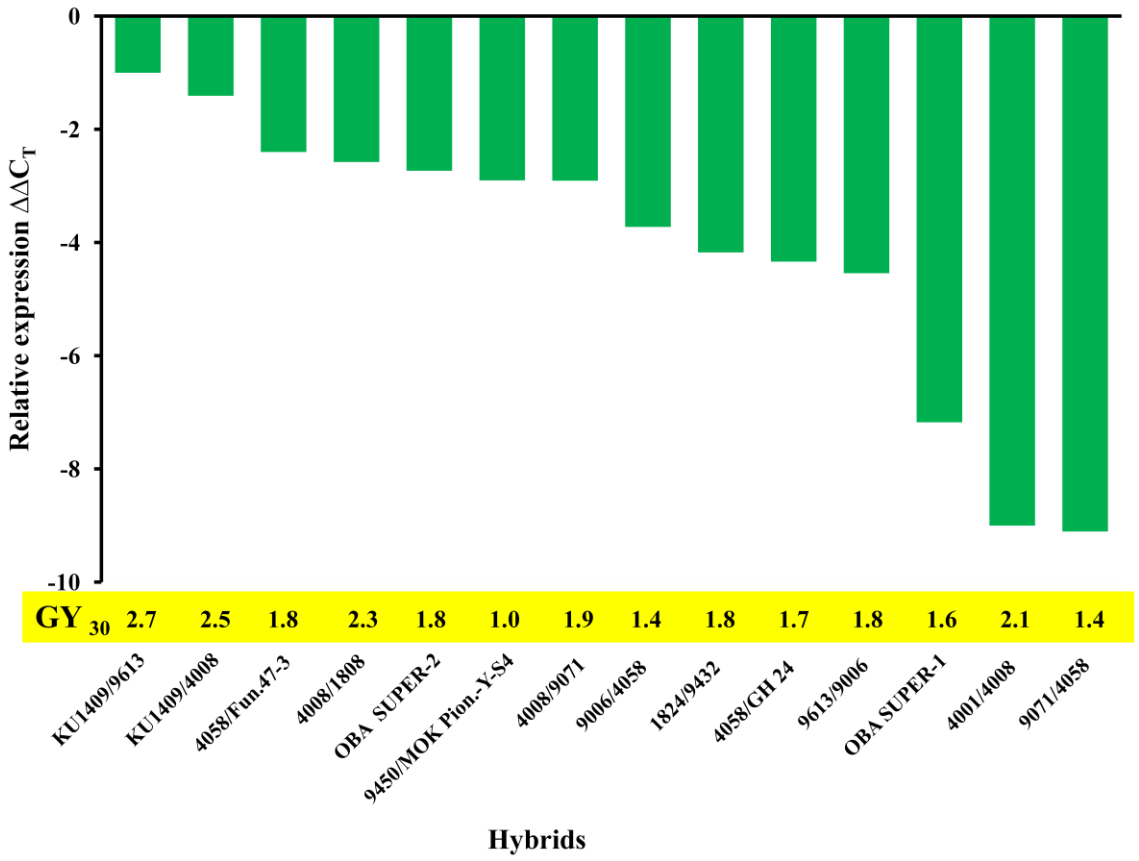

Figure 4.7. Relationship between relative L-asparaginase RNA expression in developing maize earshoots and grain yield (r=0.67*) in 14 tropical maize hybrids grown at 30 kg N/ha fertilizer at Mokwa, Nigeria. Reported as fold change normalized to GAPDH and relative to the expression level observed for the hybrid KU1409/9613. The grain yields (GY_{30}) for these hybrids from the replicated plots where samples were collected are highlighted in yellow.

Table 4.26. Correlation coefficients for developing maize earshoot L-asparaginase (ASNase) gene expression fold change, number of kernels, grain yield, N-uptake efficiency, N-utilization efficiency and N-use efficiency in 14 tropical maize hybrids grown at 30 (low-N) kg N/ha.

	Number of kernels	Grain yield	N-uptake efficiency	N-utilization efficiency	N-use efficiency
ASNase expression	0.61**	0.67**	ns	0.73***	0.49*
Number of kernels		0.74***	ns	0.48*	0.62**
Grain yield			ns	0.53**	0.82***
N-uptake efficiency				-0.58**	ns
N-utilization efficiency					0.55**

*, **, ***: significant at 0.10, 0.05, 0.01 probability levels, respectively.

CHAPTER 5
DISCUSSION

The fourteen hybrids used in this study exhibited significant genetic differences in grain yield, N-use and its related traits. Genotype × nitrogen rate interaction was significant for grain yield and N-use related traits, indicating that the hybrids differed in their response pattern to N. The interaction produced a difference in rank-order of the hybrids under the different N levels. Previous studies (Kling *et al*., 1997; Oikeh *et al*., 1998; Akintoye *et al*., 1999) have reported the lack of genotype × nitrogen rate interaction for grain yield in tropical maize, which contrasts with the result obtained in this study. The findings in the present study agrees with the results of Agrama *et al*. (1999) and Worku *et al*. (2007) in tropical maize, Bertin and Gallais (2000) in European maize, and O'Neill *et al*. (2004) in temperate maize. The significant genotype × nitrogen rate interaction obtained in the present study possibly resulted from the inclusion of hybrids selected for different responses to low-N application. The genotypic differences observed in grain yield at the same level of N fertilizer application could be attributed to differences among the maize hybrids for N-uptake and utilization (Pollmer *et al*., 1979; Beauchamp *et al*., 1976).

Reduction in maize grain yields due to N-stress obtained in this study is consistent with previous studies (Lemcoff and Loomis, 1986; Lafitte and Edmeades, 1994b; Uhart and Andrade, 1995b; Akintoye *et al*., 1999; Bänziger *et al*., 1999; Bertin and Gallais, 2000; Kamara *et al*., 2005; D'Andrea *et al*., 2006; Coque and Gallais, 2007b; Worku *et al*., 2007; de Souza *et al*., 2008; Cirilo *et al*., 2009). The observed 35% reduction in grain yield under low-N is comparable with the results of Bänziger *et al*. (1999) and Bertin and Gallais (2000) who reported 40% and 38% reduction in maize grain yield under low-N, respectively. Among yield components, kernel number was the most severely affected by N-stress with a 21% reduction, whereas kernel weight was reduced by 2% at low-N. Bertin and Gallais (2000) reported a 32% and 9% reduction for kernel number and kernel weight, respectively.

Other previous studies showed that the yield component that is most severely affected by N-stress in mauze is kernel number (Tollenaar, 1977; Uhart and Andrade,

1995b). However, contradictory responses have been reported for the effect of N-stress on kernel weight. While Muchow (1994) reported a reduction in kernel weight due to N-stress, Purcino *et al*. (2000) reported that kernel weight was not affected. Both genotypic differences (Smiciklas and Below, 1990) and the intensity of N-stress (Lemcoff and Loomis, 1986) have been suggested as possible causes of the contradictory reports. The result of this study agrees with a previous report (Coque and Gallais, 2007b) that a stress resulting in grain yield reduction higher than 20% has a greater effect on kernel number than on kernel weight. As the number of ovules initiated in maize is not limiting (Lemcoff and Loomis, 1986), the effect of N-stress on kernel number results from embryo abortion occurring within the first two weeks of ovule fertilization (Weiland and Ta, 1992; Uhart and Andrade, 1995b; Below *et al*., 1981; Bertin and Gallais, 2000). In fact, the carbon and N assimilate requirement of embryos immediately after ovule fertilization is high (Below *et al*., 2000).

In the present study, N accumulation after silking was reduced by 62% under low-N. Remobilization of N to meet kernel demand is therefore required under N stress conditions (Ta and Weiland, 1992; Rajcan and Tollenaar, 1999b). In the present study, the proportion of N remobilized ranged from 38 to 64% (mean = 53%) under low-N and from 22 to 51% (mean = 37%) under high-N. Studies by Gallais and Coque (2005) and Coque *et al*. (2008) had previously indicated that the proportion of N remobilized vary from 30 to 70% depending on genotype and environment. Furthermore, results of the study reported by Coque and Gallais (2007b) showed that more N was remobilized under low-N. The high N transfer observed in this study under low-N was only accompanied by a 2% (1.5 g/plant) dry matter transfer, a result consistent with the report of Gallais and Coque (2005) that such stover N transfer is barely accompanied by dry matter transfer.

N-use efficiency and its two primary component traits decreased from low-N to high-N, with significant genotypic differences at low-N and high-N. This is consistent with results from previous studies (Akintoye *et al*., 1999; Bertin and Gallais, 2000; Uribelarrea *et al*., 2007; de Souza *et al*., 2008). Uribelarrea *et al*. (2007) evaluated maize hybrids at six N rates and reported that N-use, N-uptake and N-utilization efficiencies were negatively related to N availability. Akintoye *et al*. (1999), who studied tropical maize materials and who had the location used in this study as one of the locations used in their study, reported an average N-use efficiency of 55 kg/kg at low-N, 32 kg/kg at medium-N and 25 kg/kg at high-N. In the present study, mean N-

use efficiency was 55.4 kg/kg at low-N and 34.3 kg/kg at high-N. The means for N-utilization efficiency obtained across hybrids, 60.6 kg/kg for low-N and 42.6 kg/kg for high-N, in the present study, were similar to those reported by Worku *et al*. (2007) and de Souza *et al*. (2008) for tropical maize, and Coque and Gallais (2007b) for European maize. In the evaluation of tropical maize hybrids under low-N and high-N conditions, Worku *et al*. (2007) obtained a mean N-utilization efficiency of 54 kg/kg at low-N and 36 kg/kg at high-N. Evaluating 23 European maize hybrids, Coque and Gallais (2007b) reported a mean N-utilization of 55 kg/kg at low-N and 47 kg/kg at high-N.

N-use efficiency has been defined as the ability of a genotype to realize an above average grain yield under conditions of low-N availability (Graham, 1984 and Sattelmacher *et al*., 1994). In the present study, hybrids 4001/4008 (E1), KU1409/9613 (E4), KU1409/4008 (E2) and 4008/1808 (E5) produced similar above average yields under both high-N and low-N application but differed in their N-uptake efficiency and N-utilization efficiency. Hybrids E1 and E4 exhibited high N-uptake efficiency while hybrids E2 and E5 exhibited high N-utilization efficiency to achieve high grain yields. These results show that the hybrids employed different mechanisms to achieve high grain yield and that both N-uptake efficiency and N-utilization efficiency are important to achieving high grain yields particularly under limiting N supply. Results from the present study are in contrast to those of Moll *et al*. (1982) and Bertin and Gallais (2000) in maize, as well as Gaju *et al*. (2011) in wheat, who reported that N-utilization efficiency is more important than N-uptake efficiency under low-N conditions. The results also contrasts that of Kamprath *et al*. (1982) who reported that N-uptake efficiency is more important than N-utilization efficiency under low-N conditions. The results of the present study are consistent with those of Worku *et al*. (2007) for tropical maize, Weisler *et al*. (2001) for temperate maize and Ortiz-Monasterio *et al*. (2001) for wheat who reported that both N-utilization efficiency and N-uptake efficiency were important for optimal performance under limiting soil N conditions.

Kernel number was the grain yield component that showed the highest positive association with grain yield, a result in agreement with the results of Bänziger *et al.* (2002) and O'Neill *et al.* (2004). This indicates that the physiological mechanisms needed to maximize kernel number are crucial for enhanced grain yields in maize hybrids. The high correlation between kernel number and stover N content as well as N-uptake efficiency at silking could relate to the strong effect of assimilate supply

during periods around flowering (Paponov *et al*., 2005). This is further supported by the strong correlations between kernel number and ear leaf chlorophyll content (SPAD) at both high-N and low-N supply. Similar to the results of Coque and Gallais (2007a), the amount of N remobilization was significantly correlated with stover N content at silking as well as percentage of N remobilization under low-N and high-N, respectively, in the current study.

Yield is a function of the action and interaction among numerous plant characters usually dependent on the genotype and environment, and the identification of the most important traits related to yield is useful to the plant breeder. The results of path analysis indicated that stover N content at silking and N-use efficiency had significant direct positive effects on grain yield under both high-N and low-N supply. Furthermore, both N-uptake efficiency and N-utilization efficiency had positive and significant direct effects on N-use efficiency at both high-N and low-N. Similar results had been reported by Samonte *et al*. (2006) for rice, where path analysis revealed that N-use efficiency and N content at heading had significant direct positive effects on rice grain yield. The positive and significant association between the components of N-use efficiency and grain yield on the one hand, and grain yield and N-use efficiency on the other, is similar to the results reported by Heuberger, (1998) and Kamara *et al*. (2003) for tropical maize adapted to the conditions in the northern Guinea savanna of West Africa. Working with the US Corn-Belt germplasm, Moll *et al*. (1982) observed no significant relationship for N-uptake efficiency and only a moderate one for N-utilization efficiency with N-use efficiency. Although the relationships between grain yield and N-uptake efficiency at silking under high-N was similar to that under low-N, the observed higher level of association of grain yield with N-uptake efficiency at maturity under low-N suggests that N-uptake efficiency could play a relatively more important role to grain yield under low-N supply, a result similar to that reported by Kamprath *et al*. (1982) and Presterl *et al*. (2002). The negative correlation between N-utilization efficiency and post-silking stover N accumulation under both N conditions shows that maize genotypes absorbing high quantities of N may not necessarily utilize the absorbed N efficiently.

Asparagine is a major nitrogen metabolite and plays a crucial role in the storage and transport of nitrogen in plants (Galili *et al*., 2008). Together with glutamine, glutamate and aspartate, it functions to connect nitrogen metabolism, photosynthesis and C/N balance in plants (Galili *et al*., 2008). Asparagine is

synthesized by the transfer of an amide from glutamine and is accumulated in a wide range of tissues and under stress conditions and nutrient deficiencies, including situations where the plant is unable to support a normal level of protein synthesis (Lea *et al*., 2007). The transport of asparagine is targeted towards developing tissues and storage organs where there is a high demand for mobilized N (Sieciechovicz *et al*., 1988). The ear requires high levels of metabolites for its growth and development as well as that of developing kernel tissues.

L-asparaginase is a key factor in the Asparagine cycling pathway and catalyses the hydrolysis of asparagine to aspartic acid and ammonia (Grant and Bevan, 1994), which are important sources of organic N for cellular processes and plant growth, and its activity is known to be located in tissues undergoing rapid growth and development (Lea *et al*., 1978; Urquhart and Joy, 1981; Grant and Bevan, 1994). Expression of asparaginase genes is also found to be prominent in tissues that previously contained localized high levels of asparagine (Grant and Bevan, 1994).

The observed down-regulation of L-asparaginase expression in the developing cob of all other hybrids relative to the highest yielding hybrid (E4) under low-N in the present study, suggests an increased asparagine synthesis and/or a reduced asparagine catabolism in the former leading to asparagine accumulation relative to aspartate and glutamine precursors. The resulting low yield is thus a consequence of the low level of metabolite supply needed for the growth and development of the cob tissue and kernels, due to reduced N remobilization from the cob to the kernels. This could be indicative of the importance of a reduced flux to asparagine for higher grain yields under limiting N condition. Since the cob serves as a temporary storage organ and a conduit for nutrients to the developing kernels (Crawford *et al*., 1982), there was the need for enhanced L-asparaginase activity to facilitate the release of metabolites for the growth and development of kernel tissues. In Arabidopsis, higher N remobilization from vegetative sinks to the seeds has been reported under low nitrogen conditions (Lemaître *et al*. 2008). In legumes and non-leguminous species including maize, N deficiency has been shown to result in a dramatic increase in xylem aspartate and a decrease in asparagine (do Amarante *et al*., 2006), suggesting that an increased asparagine catabolism, a consequence of increased asparaginase activity, is very important under limiting N conditions. Similar decreases in the asparagine content under limited N conditions were reported for rice leaves and roots (Kawachi *et al*., 2002), Arabidopsis leaves and seeds (Lemaître *et al*., 2008) and maize developing

earshoots (Seebauer *et al*., 2004). Seebauer *et al*. (2004) also reported the responsiveness of asparagine to glutamine ratio to N supply, which could serve as a signal of source plant nitrogen status to the kernel sinks. Evidences in literature (Lohaus *et al*., 1998; Seebauer *et al*., 2004; Uribelarrea *et al*., 2007) indicate that the Asparagine cycling pathway plays a key role in maize N-use efficiency, especially under N stress. Studies in maize (Seebauer *et al*., 2004; Cañas *et al*., 2009) and wheat (Howart *et al*., 2008) have demonstrated the importance of sink organs such as the ear in maize and spike in wheat in controlling N remobilization and partitioning for storage protein synthesis.

Since the expression of glutamine synthetase was equally up-regulated in the three N-use efficient hybrids relative to the N-use inefficient reference, it is plausible that this was necessary to recapture the ammonia produced by L-asparaginase activity into glutamine for amino acid biosynthesis. This prevents ammonium toxicity and enhances the re-assimilation of released nitrogen (Grant and Bevan 1994). Increased glutamine synthetase activity has been reported in Arabidopsis under limiting N conditions (Lemaître *et al*., 2008).

Hybrid 4001/4008, which was one of the highest yielding hybrids at both high-N and low-N and was N-efficient, N-responsive and had significantly lower L-asparaginase gene expression in comparison with other hybrids having similar response to N. This result suggests a different mechanism for N management in the early developmental stages of reproductive sink growth in this hybrid. Genetic and environmental variations affect many physiological and molecular traits related to N metabolism in the cob and developing kernels of maize (Cañas *et al*., 2009).

The significant and positive correlation among N-utilization efficiency, grain yield and L-asparaginase gene expression reflect the important role of L-asparaginase gene in N-utilization which is critical for efficient use of N especially under limiting N conditions. The enhanced L-asparaginase gene activity observed in most of the N-efficient and N-responsive hybrids has potential for use as a good indicator of N-utilization.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Maize plays an important role in the nutition of people of sub-Saharan Africa. However, its production in the sub-region is constrained by several factors, among which are low soil nitrogen (N) and sub-optimal N fertilizer application. The latter factor results from limited availability and cost of inorganic N fertilizers.

Genotypes with improved nitrogen use efficiency (NUE), defined as grain yield per unit N supplied by fertilizer and soil, would be beneficial to low-input agriculture. Such genotypes would have improved grain yields under low soil N and optimal N availability. NUE is a function of two main agronomic traits: N-uptake efficiency (NUpE), defined as the total N-uptake per unit N supplied by the soil, and N-utilization efficiency (NUtE), defined as the grain yield produced per unit of N absorbed from the soil.

Combining both physiological and genetic information will facilitate genetic improvement in maize NUE. Therefore, information is required on the relative importance of NUpE and NUtE to NUE under sub-optimal and adequate N availability. Furthermore, gene expression differences in the developing cob tissue in response to applied N could be indicative of the potential of identified genes to initiate kernel formation and development, and its relationships with agronomic and physiological traits under limited N availability could provide information on NUE. This study was therefore carried out to: (i) evaluate genetic variation for N-use efficiency among selected tropical maize hybrids, (ii) establish the relative importance of N-uptake efficiency and N-utilization efficiency to N-use efficiency, (iii) determine agronomic and physiological traits that could be used to optimize selection for high grain yield under both N-stress and non N-stress conditions, (iv) identify genes associated with N responses in developing earshoots of selected tropical maize hybrids, and (v) identify those genes that are promising for marker assisted-selection and breeding for efficient use of N.

Fourteen tropical maize hybrids differing in grain yield (GY) under low-N conditions were grown under no-N (0 kg N/ha), low-N (30 kg N/ha) and high-N (90

kg N/ha) levels from 2006 to 2008 in a randomized complete block design with four replications. Analysis of variance was carried out on data collected on GY and its components, NUE, NUpE, NUtE and N-related traits. Microarray technique using Maize Oligonucleotide array slides containing ~57000 probes were used to identify differentially expressed genes in developing cob tissues harvested from three N-use efficient hybrids compared to an N-use inefficient reference hybrid. Differentially expressed genes were validated by quantitative Real Time Polymerace Chain Reaction (qRT-PCR).

The study showed that:

i. Genetic variation exists among the tropical maize hybrids studied for N-use efficiency and its components.

ii. In the tropical maize hybrids studied, both N-uptake efficiency and N-utilization efficiency contribute to high grain yield under low-N and high-N. Although the relative importance of both traits to N-use efficiency varied with genotype and level of N, the negative and significant association of the traits may not allow for simultaneous improvement in both traits.

iii. The hybrids 4001/4008, KU1409/9613, KU1409/4008, 4008/1808, 4058/Fun 47-4 and 1824/9432 were found to be both N-efficient and N-responsive exhibiting above average yields at both levels of N.

iv. N-utilization efficiency and post-silking stover N accumulation were negatively and significantly related under both low-N and high-N suggesting that maize hybrids absorbing high quantities of N do not necessarily utilize it efficiently.

v. The amount of N remobilization was positively and significantly correlated with stover N content at silking under both high-N and low-N. Under low-N, 53% of stover N content at silking was remobilized while 37% was remobilized at high-N. Hybrids varied in their capacity to remobilize N under both high-N and low-N.

vi. Stover N content at silking and N-use efficiency are the most important traits affecting maize grain yield and could be used to select for high grain yield under both high-N and low-N.

vii. Only 163 genes representing about 0.3% of the genes on the Maize Oligonucleotide array were differentially expressed in the three hybrids under low-N application; of the key genes linked to the Asparagine cycling pathway,

only L-asparaginase (MZ00019244) and glutamine synthetase (MZ00027609) were differentially expressed in the three selected N-efficient hybrids in comparison with the N-inefficient and N-non responsive reference check hybrid.

viii. L-asparaginase mRNA expression in developing cob tissue is positively and significantly related with N-utilization efficiency and grain yield, and thus could serve as a valuable tool for predicting maize genotypes with good N-utilization efficiency and superior grain yield under low soil N.

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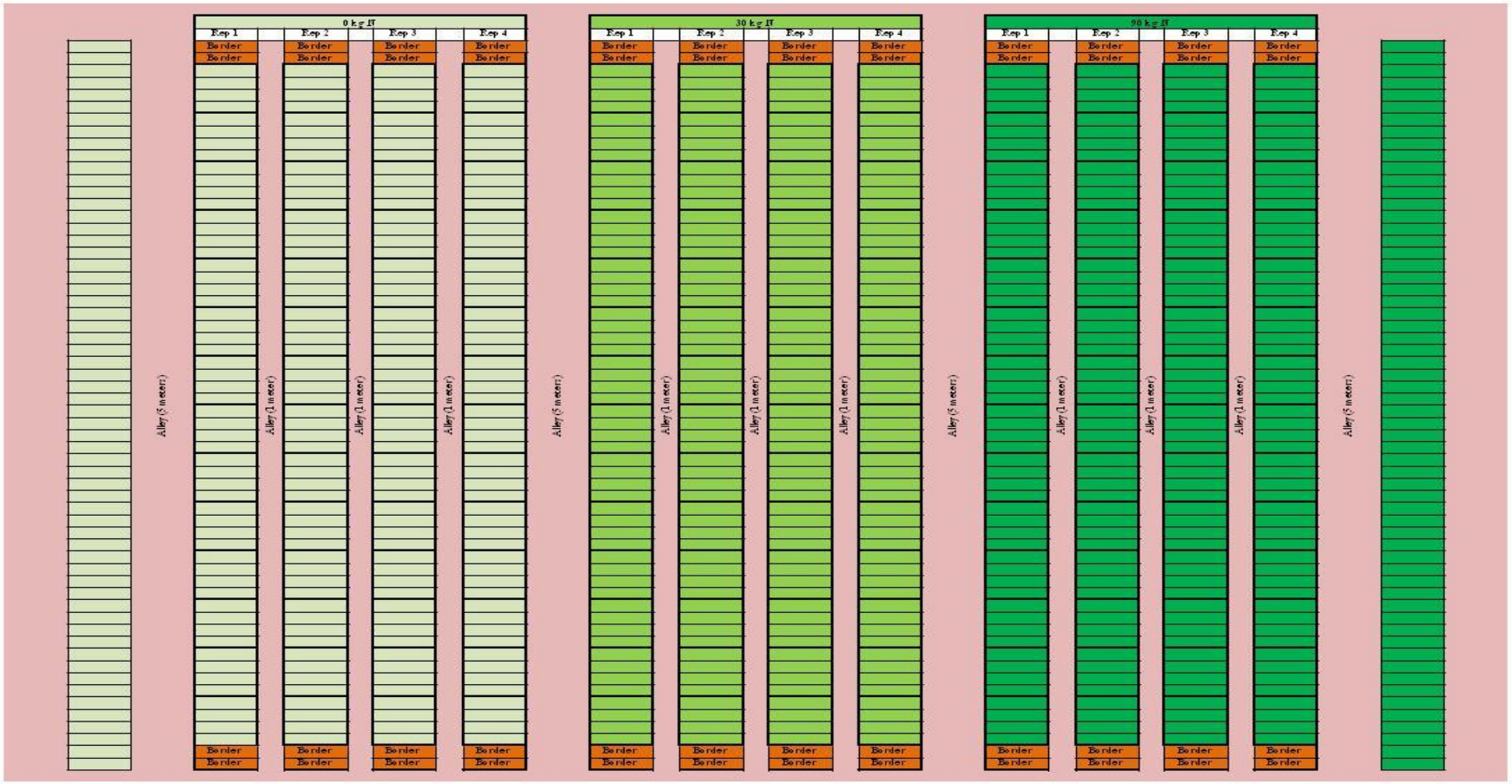
APPENDICES

APPENDIX 1. Field layout showing how the experiment was set up on the field



APPENDIX 2. Final Microarray set-ups for earshoot samples collected at 30kg N ha^{-1}

Genotype	Rep	Slide number	Cy3	Cy5	Slides	Tubes
4001/4008 G3	1	171	R (low)	1a (high)	A	1
	1	172	1a (high)	R (low)	A	2
	2	175	R (low)	1b (high)	A	3
	2	176	1b (high)	R (low)	A	4
KU1409/4008 G2	1	169	R (low)	2a (high)	A	5
	1	170	2a (high)	R (low)	A	6
	2	173	R (low)	2b (high)	A	7
	2	174	2b (high)	R (low)	A	8
KU1409/9613 G1	1	217a	R (low)	3a (high)	A	9
	1	218	3a (high)	R (low)	A	10
	2	219	R (low)	3b (high)	A	11
	2	220	3b (high)	R (low)	A	12
4001/4008 G3	1	150	R (low)	1a (high)	B	13
	1	151	1a (high)	R (low)	B	14
	2	154	R (low)	1b (high)	B	15
	2	155	1b (high)	R (low)	B	16
KU1409/4008 G2	1	148	R (low)	2a (high)	B	17
	1	149	2a (high)	R (low)	B	18
	2	152	R (low)	2b (high)	B	19
	2	153	2b (high)	R (low)	B	20
KU1409/9613 G1	1	193	R (low)	3a (high)	B	21
	1	194	3a (high)	R (low)	B	22
	2	195	R (low)	3b (high)	B	23
	2	196	3b (high)	R (low)	B	24

Key

R (Reference) genotype = Oba Super-1

R = low yield, low asparaginase

G1 = high yield, high asparaginase

G2 = high yield, high asparaginase

G3 = high yield, low asparaginase

APPENDIX 3. 163 genes showing significant differential expression across all the three test hybrids

ID	Sequence Description	Fold change			E value
		G1/M	G2/M	G3/M	
MZ00001598	Unknown protein	4.99	7.62	6.45	NA
MZ00001951	Unknown protein	0.38	0.48	0.23	0
MZ00002534	Cytochrome p450	0.15	0.24	3.72	0
MZ00003821	Auxin-repressed protein	0.17	0.20	0.21	2.36E-04
MZ00003834	Oxysterol-binding protein 1	4.38	8.06	2.27	1.59E-164
MZ00004120	Phospholipid transfer protein - maize {Zea mays;}	0.37	0.20	1.75	NA
MZ00004581	Unknown protein	0.36	0.06	0.16	NA
MZ00004641	Unknown protein	2.92	2.50	2.28	NA
MZ00004647	Cyclin-dependent protein	0.56	0.31	1.83	8.02E-32
MZ00004859	Unknown protein	0.35	0.19	0.21	1.15E-08
MZ00008937	Unknown protein	0.43	0.34	0.41	5.03E-67
MZ00009106	nucellin-like aspartic protease	0.18	0.19	2.46	3.64E-42
MZ00013727	Unknown protein	0.41	0.10	0.28	NA
MZ00013729	Unknown protein	0.32	0.37	0.21	NA
MZ00013813	Ozone-responsive stress-related	2.83	2.75	3.35	7.49E-42
MZ00013901	Auxin-repressed kda protein	0.10	0.09	0.21	3.21E-50
MZ00013972	NADH-ubiquinone oxidoreductase 18 kda subunit	4.99	11.29	7.43	1.41E-19
MZ00014019	Pre-mRNA-splicing factor cwc15	0.25	14.46	5.04	2.49E-74
MZ00014665	Alanine-glyoxylate aminotransferase mitochondrial expressed	0.43	0.32	0.46	0
MZ00014709	HAT dimerisation domain-containing	0.17	0.13	0.28	7.83E-54
MZ00014841	Class i chitinase	2.56	14.05	10.74	4.19E-142
MZ00015075	Actin-like protein 3	0.46	0.36	0.46	2.44E-134
MZ00015111	BTH-induced protein phosphatase 1 {Oryza sativa (indica cultivar-group);}	0.29	0.16	0.22	0
MZ00015290	MADs-box transcription factor 32	0.49	0.15	0.17	2.74E-52
MZ00015345	Unknown protein	0.37	0.18	0.34	1.85E-13
MZ00015509	Harpin-induced protein 1 family (HIN1)-like {Oryza sativa (japonica cultivar-group);}	0.35	0.43	1.85	1.10E-97
MZ00015611	Retrotransposon unclassified	3.42	7.63	3.99	1.01E-28

APPENDIX 3. continued

ID	Sequence Description	Fold change			E value
		G1/M	G2/M	G3/M	
MZ00015918	Kid-containing protein	0.32	0.15	0.36	5.64E-71
MZ00016003	Methyl chloride transferase	0.42	0.11	0.47	1.33E-133
MZ00016016	USP family protein	2.58	4.93	1.93	9.75E-52
MZ00016093	Mitochondrial ribosomal protein l11	8.18	20.15	6.26	1.72E-43
MZ00016260	Zinc finger family expressed	0.18	0.06	0.05	1.45E-165
MZ00016597	Putative histone H2A {Oryza sativa (japonica cultivar-group);}	2.87	6.32	3.89	9.86E-38
MZ00016646	Osmotin-like protein {Oryza sativa (japonica cultivar-group);}	0.16	0.18	0.46	3.02E-133
MZ00016691	Protein kinase	4.25	2.10	2.01	3.83E-88
MZ00017606	Putative LRR receptor-like kinase 2 {Oryza sativa (japonica cultivar-group);}	2.17	2.52	2.05	5.51E-152
MZ00017848	Leucine-rich repeat-like protein {Oryza sativa (japonica cultivar-group);}	0.12	2.98	0.40	0
MZ00017849	Ras-related protein rab11d	3.22	2.29	2.50	3.51E-115
MZ00017851	O-methyltransferase ZRP4 (EC 2.1.1.-) (OMT). {Zea mays;}	0.43	0.37	4.38	0
MZ00018193	Unknown protein	0.28	0.24	2.25	NA
MZ00018308	Unknown protein	3.07	3.63	4.32	7.14E-31
MZ00018371	Unknown protein	0.32	0.39	0.53	0
MZ00018736	Putative bHLH transcription protein {Oryza sativa (japonica cultivar-group);}	5.08	14.90	8.28	NA
MZ00019168	Small heat-shock	1.72	2.13	0.43	3.18E-85
MZ00019244	l-asparaginase 2	2.91	2.90	0.46	1.98E-59
MZ00019364	Flavonoid 3 -hydroxylase	0.35	0.18	2.42	7.73E-173
MZ00019868	Sant myb protein	2.45	2.18	2.52	2.65E-36
MZ00019969	Blue copper protein	0.40	0.19	3.22	1.57E-25
MZ00020079	Unknown protein	0.46	0.10	0.20	NA
MZ00020563	F-box family protein	2.31	1.98	0.53	7.91E-138
MZ00020679	Unknown protein	1.96	0.53	5.03	NA
MZ00020721	Unknown protein	3.27	2.96	3.44	2.61E-31
MZ00021108	Coatomer protein subunit beta 2 (beta prime)	5.45	2.17	2.20	4.32E-14
MZ00021146	Unknown protein	2.97	6.37	4.00	NA
MZ00021210	Chitinase	2.03	2.13	1.70	5.02E-169
MZ00021298	Unknown protein	0.37	0.25	0.19	3.35E-81
MZ00021377	Snf1-related protein kinase regulatory subunit beta-1	0.36	0.25	0.29	2.30E-140
MZ00021437	Gibberellin receptor gid1l2	0.55	0.46	2.08	0
MZ00021579	3-oxoacyl-synthase iii	2.59	3.33	2.13	1.86E-62
MZ00021589	Histone h2b	0.52	0.56	0.34	5.70E-55

APPENDIX 3. continued

ID	Sequence Description	Fold change			E value
		G1/M	G2/M	G3/M	
MZ00021602	Unknown protein	5.98	5.36	0.27	NA
MZ00023572	O-methyltransferase	0.22	0.23	0.04	3.26E-79
MZ00023590	ADP-ribosylation factor 1	2.16	2.69	2.71	5.34E-99
MZ00023839	Unknown protein	0.14	0.42	0.08	4.59E-10
MZ00023848	Farnesylated protein 1	3.34	3.07	2.49	5.23E-84
MZ00023884	Unknown protein	0.42	0.44	0.39	NA
MZ00023889	Putative NADH dehydrogenase {Oryza sativa (japonica cultivar-group);}	3.95	3.29	2.71	1.47E-161
MZ00023959	Unknown protein	0.34	0.52	0.52	8.53E-21
MZ00024031	Serine carboxypeptidase family expressed	2.80	5.44	3.17	6.78E-111
MZ00024113	Auxin response factor expressed	1.76	1.70	0.56	0
MZ00024314	O-methyltransferase	0.07	0.17	3.73	0
MZ00024315	Manganese superoxide dismutase	1.94	2.59	2.30	1.20E-122
MZ00025141	Protein phosphatase 2a	3.82	3.75	2.14	6.22E-104
MZ00025155	Carboxyphosphonoenolpyruvate mutase	14.16	86.55	23.49	0
MZ00025298	Leafy hull sterile 1	0.29	0.05	0.23	8.05E-54
MZ00025324	Transferring glycosyl	9.08	12.86	6.40	4.18E-35
MZ00025407	Polyamine oxidase	2.82	2.87	2.51	0
MZ00025431	Bowman birk trypsin inhibitor	0.31	0.17	0.12	1.91E-111
MZ00025484	14-3-3-like protein	4.80	9.98	9.57	1.98E-29
MZ00025532	MYB family transcription expressed	2.27	1.92	0.45	1.64E-140
MZ00025781	Unknown protein	1.81	2.32	1.91	0
MZ00025882	Unknown protein	0.42	0.48	0.35	4.56E-54
MZ00025924	Mitochondrial glycoprotein	1.94	2.04	2.64	4.22E-114
MZ00025961	Latex cyanogenic beta glucosidase	2.09	2.11	2.30	1.40E-89
MZ00025987	NHLrepeat-containing protein	2.13	2.16	0.46	0
MZ00026025	Geko1-like protein	0.43	0.54	0.36	0
MZ00027034	Trehalose-6-phosphate synthase	0.55	0.48	0.43	3.82E-169
MZ00027105	Bowman-birk type trypsin inhibitor	0.38	0.18	0.22	2.17E-68
MZ00027136	F-box domain containing expressed	2.50	2.92	0.22	3.74E-145
MZ00027383	Ferredoxin- chloroplast	19.90	132.35	11.99	2.43E-82
MZ00027410	alpha-l-fucosidase 2 precursor	0.50	0.26	1.93	2.46E-166
MZ00027467	Integral membrane	0.29	0.11	2.27	0
MZ00027609	Glutamine synthetase	2.14	4.29	2.42	0
MZ00027798	Metal tolerance protein	3.56	4.54	4.26	0
MZ00027896	Unknown protein	0.45	0.17	2.39	8.74E-17
MZ00027943	ATP binding	1.86	1.79	1.82	1.35E-28
MZ00028108	Peroxidase 1 precursor	0.34	0.08	2.56	1.56E-180

APPENDIX 3. continued

ID	Sequence Description	Fold change			E value
		G1/M	G2/M	G3/M	
MZ00028359	Protein kinase	1.90	2.14	0.59	3.78E-178
MZ00028466	Precursor of carboxylase h-protein glycine decarboxylase complex	2.35	2.32	2.46	2.48E-32
MZ00028495	Protein kinase	4.14	4.30	2.91	3.08E-37
MZ00028498	Peroxidase 2 precursor	0.47	0.32	1.92	1.07E-129
MZ00029961	Ripening-related protein 3 flags: precursor	0.30	3.08	0.41	1.96E-70
MZ00030565	DNA binding protein	0.34	0.13	0.28	1.27E-15
MZ00030677	Unknown protein	0.32	0.19	4.15	1.91E-20
MZ00030949	Stearoyl-acyl-carrier protein desaturase	3.90	14.10	14.46	8.91E-46
MZ00032349	Unknown protein	0.51	0.32	0.37	2.91E-06
MZ00032607	Unknown protein	0.22	0.08	0.20	NA
MZ00033388	Homeobox protein rough sheath 1	0.48	0.40	0.45	1.50E-130
MZ00033524	Putative organic solute transporter {Oryza sativa (japonica cultivar-group);}	0.36	0.16	0.23	NA
MZ00033711	NAC1 transcription factor	0.28	0.38	0.39	4.26E-162
MZ00034150	non-cyanogenic beta-glucosidase precursor	0.05	0.53	0.18	0
MZ00035649	Bowman birk trypsin inhibitor	0.22	0.43	0.21	5.86E-111
MZ00035947	NAC1 transcription factor	0.35	0.35	0.41	4.26E-162
MZ00036085	Tonoplast membrane integral protein 4-3	0.44	0.41	0.46	2.94E-41
MZ00036098	Metallothionein-like protein	0.13	0.19	3.03	1.09E-10
MZ00036108	Rhicadhesin receptor precursor	0.12	0.45	2.08	1.90E-108
MZ00036168	Calcineurin b-like protein	0.50	0.16	0.35	2.18E-86
MZ00036180	Unknown protein	0.40	0.19	0.49	NA
MZ00036215	Unknown protein	1.95	2.89	2.42	8.91E-22
MZ00037083	Metallothionein-like protein	0.27	0.17	4.78	8.54E-33
MZ00037123	Unknown protein	0.47	0.36	3.72	6.79E-62
MZ00037136	Proline-rich protein	2.93	9.39	4.30	1.30E-28
MZ00037287	Unknown protein	2.41	3.09	2.90	NA
MZ00037498	Unknown protein	2.48	4.42	4.08	0
MZ00037552	Unknown protein	2.46	8.64	6.21	NA
MZ00037902	UDP-galactose 4-epimerase-like protein	0.36	0.24	0.24	8.28E-65
MZ00038585	Unknown protein	3.00	2.03	2.04	NA
MZ00039256	Unknown protein	0.48	0.11	2.09	NA
MZ00039449	Auxin-repressed kda protein	0.08	0.12	0.26	1.04E-48
MZ00040421	Cold regulated protein	0.43	0.11	0.19	8.08E-47
MZ00041198	Unknown protein	0.43	0.09	0.24	NA
MZ00041204	Type 1 non specific lipid transfer protein precursor	0.20	0.23	4.12	1.38E-40

APPENDIX 3. continued

ID	Sequence Description	Fold change			E value
		G1/M	G2/M	G3/M	
MZ00042051	Unknown protein	2.05	2.18	1.76	3.07E-51
MZ00042059	Thioredoxin-like 1	0.22	0.32	0.40	7.52E-111
MZ00042098	Unknown protein	0.35	0.27	0.46	NA
MZ00042156	Homeobox protein rough sheath 1	9.06	8.94	3.57	2.18E-131
MZ00042215	beta-expansin 1a precursor	0.36	0.11	3.01	3.49E-150
MZ00042403	Ethylene-insensitive3-like 1 expressed	0.19	0.06	0.08	9.00E-53
MZ00042450	Vamp protein sec22	0.47	0.38	2.77	2.82E-86
MZ00042579	bHLH transcription factor PTF1 {Oryza sativa;}	2.36	1.71	3.03	1.99E-111
MZ00042881	60s ribosomal protein l37a	2.26	3.04	2.21	9.50E-47
MZ00042940	Unknown protein	0.50	0.19	0.18	1.21E-25
MZ00042949	Unknown protein	0.43	0.27	0.24	4.31E-19
MZ00042973	O-methyltransferase	0.22	0.36	0.18	0
MZ00043215	Unknown protein	0.16	0.18	0.18	1.12E-13
MZ00043222	Unknown protein	0.30	0.24	0.41	2.85E-17
MZ00043223	Unknown protein	0.34	0.06	0.21	NA
MZ00043226	Unknown protein	0.45	0.08	0.18	NA
MZ00043400	Stem-specific protein tsjt1	0.08	0.17	0.23	1.98E-116
MZ00043421	Chaperone protein dnaJ-like {Oryza sativa (japonica cultivar-group);}	0.34	0.25	0.42	9.36E-17
MZ00043543	non-green plastid inner envelope membrane protein	2.38	2.01	2.68	2.89E-146
MZ00043644	Ethylene response factor	0.13	0.10	0.46	1.14E-126
MZ00043822	Unknown protein	2.50	2.64	2.40	1.56E-83
MZ00044877	Lipoxygenase expressed	0.42	0.33	2.40	1.49E-75
MZ00045978	Unknown protein	0.53	0.18	0.18	NA
MZ00046019	bHLH transcription factor(GBOF-1)-like {Oryza sativa (japonica cultivar-group);}	3.71	2.56	3.80	1.15E-29
MZ00046021	Unknown protein	0.52	0.33	0.45	NA
MZ00046186	Unknown protein	0.53	0.40	0.28	4.66E-09
MZ00046397	Protein phosphatase 2c	0.19	0.45	0.40	8.13E-74
MZ00053792	Unknown protein	4.41	11.40	7.52	NA
MZ00055283	ATPase inhibitor	0.37	0.12	0.23	5.58E-20
MZ00056765	Prefolding subunit 2	2.10	3.74	2.83	1.10E-37
MZ00057236	NAC1 transcription factor	0.24	0.40	0.32	5.70E-162

APPENDIX 4. Primers used for real-time qRT-PCR

ID	Sequence Description	Primer Sequences	
MZ00004043	Sucrose synthase 1 (EC 2.4.1.13)	*Forward*	ATGTCCGAGTTGGGGTATTTGGTT
		Reverse	TCTCTCGTTTTGATGTCTGGCCATA
MZ00017849	Ras-related protein rab11d	*Forward*	GTCAAGACGGTCTGGAAGGCATT
		Reverse	TGATCATGCTGGTTGGCAACAA
MZ00019244	l-asparaginase 2	*Forward*	GCCTCCAGCTCACTCCCAGATG
		Reverse	TCGACTACTGCGTCAAGGAGCG
MZ00024296	1-aminocycloprotein-1-carboxylase oxidase	*Forward*	AGCTCCACATCGTGTGCCAGTACTA
		Reverse	TCCACGCTCTTGTACCGGTCAT
MZ00025484	14-3-3-like protein	*Forward*	TGAAGTTTCTGTCCGTCTCGGGTT
		Reverse	ACGCTTCAGTCACCGAACATGATTA
MZ00027609	Glutamine synthetase	*Forward*	AGCTGAGGTGCCATGGTACGGTA
		Reverse	CAGGCTTTGTAGTGGGCGTCAA
MZ00042156	Homeobox protein rough sheath 1	*Forward*	CTCTCGCTCGCCGATGGTAAAT
		Reverse	CCTCCTTTGGGAGCTTCCCTTT
MZ00021642	Putative bZIP transcription factor RF2b	*Forward*	CGTAGCCGCTCGATCTCCTTCTT
		Reverse	CGCAGAGGTCACGAGTCAGGAA
MZ00042579	bHLH transcription factor PTF1 {Oryza sativa;}	*Forward*	TAGGGTAGGAGCTCCAGGAGCAGTT
		Reverse	TTTCTGGGTGGATATTGCGGAA