MICHELLE GUITTON COTTA

MOLECULAR MECHANISMS IN THE FIRST STEP OF ABA-MEDIATED RESPONSE IN *Coffea* ssp

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para a obtenção do título de Doutor.

Orientador

Dr. Alan Carvalho Andrade

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MECANISMOS MOLECULARES DA PRIMEIRA ETAPA DA RESPOSTA MEDIADA POR ABA EM *Coffea* ssp

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“It is not the strongest or the most intelligent who will survive but those who can best manage change.”

Charles Darwin

ABSTRACT

Abscisic acid (ABA) is a phytohormone universally conserved in land plants which coordinates several aspects of the plant response to water deficit such as root architecture, seed dormancy and regulation of stomatal closure. A mechanism of ABA signal transduction has been proposed, evolving intracellular ABA receptors (PYR/PYL/RCARs) interacting with PP2Cs phosphatases and SnRK2 protein kinases. The goal of this study was to identify and characterize for the first time the orthologs genes of this tripartite system in *Coffea*. For this purpose, protein sequences from *Arabidopsis*, citrus, rice, grape, tomato and potato were chosen as query to search orthologous genes in the Coffee Genome Hub (http://coffee-genome.org/). Differential expression in tissues as leaves, seeds, roots and floral organs was checked through *in silico* analyses. *In vivo* gene expression analyses were also performed by RT-qPCR in leaves and roots of drought-tolerant (D^T 14, 73 and 120) and drought-susceptible (D^S 22) *C. canephora* Conilon clones submitted (or not) to drought. The expression profiles of the tripartite system *CcPYL-PP2C-SnRK2* genes were also analyzed in leaves of *C. arabica* (*Ca*) and *C. canephora* (*Cc*) plants grown under hydroponic condition and submitted to ABA exogenous treatment (500 µM). This approach allowed the identification and characterization of 24 candidate genes (9 *PYL/RCARs*, 6 *PP2Cs* and 9 *SnRK2s*) in *Cc* genome. The protein motifs identified in predict coffee sequences enabled characterize these genes as family's members of PYL/RCARs receptors, PP2Cs phosphatases or SnRK2 kinases of the ABA-dependent response pathway. These families were functionally annotated in the *Cc* genome. *In vivo* analyses revealed that eight genes were up-regulated under drought conditions in both leaves and roots tissues. Among them, three genes coding phosphatases were expressed in all (D^T and D^S) clones therefore suggesting that they were activated as a general response to cope with drought stress. However, two other phosphatase coding genes were up-regulated only in the D^T clones, suggesting that they constitute key-genes for drought tolerance in these clones. The D^T clones also showed differential gene expression profiles for five other genes thus reinforcing the idea that multiple biological mechanisms are involved in drought tolerance in *Cc*. In response to exogenous ABA, 17 genes were expressed in leaves of *Cc* and *Ca* plants. Several genes were differentially expressed in the D^T clone 14 either in control condition or after 24h with ABA treatment. Under control condition, five genes were higher expressed as in the *Cc* as in *Ca* D^T plants. The kinase *CcSnRK2.6* was highlighted as gene specifically expressed in the *Cc* plants (D^T and D^S) after 72h of ABA treatment. Overall, it was observed that ABA signaling pathway is delayed in the D^S *C. arabica* Rubi. Those molecular evidences corroborated with microscopies analyses which showed that the D^T clone 14 was more efficient to control the stomatal closure than other coffee plants in response to ABA treatment. All these evidences will help us to identify the genetic determinism of drought tolerance through ABA pathway essential to obtain molecular markers that could be used in coffee breeding programs.

Keywords: Abscisic acid. Gene expression. Drought. *Coffea canephora*. *Coffea arabica*.

RESUMO

O ácido abscísico (ABA) é um fitohormônio universalmente conservado entre as plantas o qual coordena vários aspectos de resposta ao déficit hídrico, tais como, arquitetura radicular, dormência de sementes e regulação do fechamento estomático. Um mecanismo de transdução de sinal de ABA foi proposto envolvendo os receptores intracelulares de ABA (PYR/PYL/RCARs) que interagem com as fosfatases PP2Cs e proteínas quinases SnRK2. O objetivo desse estudo foi identificar e caracterizar pela primeira vez os genes ortólogos desse sistema tripartite em *Coffea*. Sequências proteicas de *Arabidopsis*, citros, arroz, uva, tomate e batata foram escolhidas como *query* para buscar genes ortólogos no Coffee Genome Hub (http://coffee-genome.org/). A expressão diferencial em folhas, sementes, raízes e órgãos florais foi verificada por meio de análises *in silico*. As análises de expressão gênica *in vivo* foram também realizadas por RT-qPCR em folhas e raízes de clones de *C. canephora* (*Cc*) tolerantes (D^T 14, 73 e 120) e suscetíveis (D^S 22) à seca os quais foram submetidos ou não ao déficit hídrico. Os perfis de expressão desses genes foram também analisados em folhas de plantas de *Ca* e *Cc* crescidas em condição hidropônica e submetidas à tratamento com ABA exógeno (500 µM). Essa abordagem permitiu a identificação e a caracterização de 24 genes candidatos (9 *PYL/RCARs*, 6 *PP2Cs* e 9 *SnRK2s*) no genoma de *Cc*. Os motivos proteicos identificados permitiram caracterizar os respectivos genes como membros das famílias de receptores PYL/RCARs, fosfatases PP2Cs e quinases SnRK2 da via de resposta ABA-dependente. Essas famílias foram funcionalmente anotadas no genoma de *Cc*. Análises *in vivo* revelaram que oito genes foram super expressos em condição de seca em tecidos foliares e radiculares. Entre eles, três genes que codificam fosfatases foram expressos em todos (D^T e D^S) clones, consequentemente sugerindo que esses genes foram ativados como uma resposta geral para lidar com o déficit hídrico. Entretanto, dois outros genes que codificam fosfatases foram super expressos somente nos clones D^T, sugerindo que eles constituem genes-chave para a tolerância a seca nesses clones. Os clones D^T também apresentaram perfil de expressão gênica diferencial para cinco outros genes e desse modo reforçam a ideia de que múltiplos mecanismos biológicos estão envolvidos na tolerância a seca em *Cc*. Em resposta a ABA exógeno, 17 genes foram expressos em folhas de plantas de *Cc* e *Ca*. Muitos genes foram diferencialmente expressos no clone 14 D^T tanto em condição controle como após 24h de tratamento com ABA. Em condição controle, cinco genes foram mais expressos tanto nas plantas D^T de *Cc* como de *Ca*. A quinase *CcSnRK2.6* se destacou por ser expresso somente nas plantas de *Cc* (D^T e D^S) após 72h de tratamento com ABA. De forma geral, foi observado que a via de sinalização de ABA é atrasada nos *Ca* var Rubi D^S. Tais evidências moleculares corroboram com as análises microscópicas que mostram que o clone 14 D^T foi mais eficiente para controlar o fechamento estomático em resposta ao tratamento com ABA do que as outras plantas de café. Todas essas evidências irão nos ajudar a identificar o determinismo genético para a tolerância à seca por meio da via ABA-dependente essencial para obter marcadores moleculares que podem ser usados em programas de melhoramento.

Palavras-chave: Ácido abscísico. Expressão gênica. Seca. *Coffea canephora*. *Coffea arabica.*

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Chapter 1

SUMMARY

PREFACE

The *Coffea* genus belongs to Rubiacea family and contains more than 124 species (Davis et al., 2006, 2011) that represents a major agricultural commodity in world trade (ICO, 2016). This genus comprises perennial species, all native to Madagascar, Africa, the Mascarene Island and the Comoros Island. Among all species, *C. arabica* and *C. canephora* are the two economically important species. As provider of a higher quality beverage *C. arabica* is the most cultivated specie (Poncet et al., 2007). *C. arabica* is an allotetraploid (2n = 4x = 44) that was originated 1 million years from the natural hybridization of two ancestral diploid genomes, *C. canephora* and *C. eugenioides* (Lashermes et al., 1999). Due to the self-pollination of the flowers, the species is characterized by a low genetic diversity (Hatanaka et al., 1999). Conversely, *C. canephora* is a diploid species (2n = 2x = 22), it has high genetic variability and ability to adapt to various climatic conditions (Bertrand et al., 2003). However, produces a lower quality coffee, more suitable for the production of instant coffee (Hatanaka et al., 1999).

Currently, the annual world production is around 143.3 million bags (60 Kg) coffee beans (ICO, 2016), being Brazil the largest producer (30,2%). Nowadays, drought and unfavorable temperatures are the major climatic limitations for coffee production, in some marginal regions with no irrigation coffee yields may decrease as much as 80% in very dry years (Damatta; Ramalho, 2006). As a consequence of global warming, coffee-growing geographical regions could also suffer delocalization (Assad et al., 2004). Variatiations in rainfall and temperature also influences biochemical composition of beans (Mazzafera, 2007) affecting directly the final cup quality. There is genetic variability within the *Coffea* genus that could be used to increase drought tolerance and generate coffee varieties better adapted to climatic variations which has been turned into one of the priorities of many coffee research institutes (Marraccini et al., 2012). Elucidate

the genetic and molecular mechanisms of drought tolerance is essential to identificate molecular markers that could be used to speed up coffee breeding programmes (Leroy et al., 2011).

Abscisic Acid (ABA), discovered in the 1960s (Ohkuma et al., 1963; Cornforth et al., 1965) is a vital plant hormone synthesized in roots and leaves (Zhang; Davies, 1989; Thompson et al., 2007) which act as central regulator that protects plants against abiotic stresses such drought (Wasilewska et al., 2008; Soon et al., 2012). ABA can accumulate up to 10 to 30-fold in plants under drought stress relative to unstressed conditions (Leung et al., 2012). ABA has been characterized as important endogenous small molecule that mediates stress-responsive gene expression, stomatal closure, and vegetative growth modulation (Rodríguez-Gacio et al., 2009). A great deal of effort has been focused on elucidating the molecular mechanisms underlying ABA sensing and signalling over the past few decades (Umezawa et al., 2010). Recently, two independent research groups discovered novel intracelular ABA receptors, PYL/RCARs, that are involved in ABA sensing and signaling via their direct interaction with clade A PP2Cs in *Arabidopsis thaliana* (Ma et al., 2009; Park et al., 2009). With the looming prospect of global water crisis, these recent laudable success in deciphering the early steps in the signal transduction of the "stress hormone" ABA has ignited hopes that crops can be engineered with the capacity to maintain productivity while requiring less water input (Leung et al., 2012).

The core of the ABA signaling network comprises a subfamily of type 2C proteins phosphatases (PP2Cs) and three Snfl-related kinases, SnRK2.2, 2.3 and 2.6 (Umezawa et al., 2009; Fujii et al., 2009) whose activities are controlled by ABA. The current ABA signal transduction model can be described as follow: In the absence of ABA, SnRK2 kinases are inactivated by PP2Cs which physically interact with SnRK2 and dephosphorylate a serine residue in the kinase activation loop, a phosphorylation essential for kinase activity (Belin et al., 2006). ABA

binds to the ABA receptors family PYR/PYL/RCAR allowing the bounds of the receptors and the catalytic site of PP2Cs to inhibit their enzymatic activity. In turn, ABA-induced inhibition of PP2Cs leads to SnRK2 activation by activation loop autophosphorylation (Boudsocq et al., 2007; Soon et al., 2012).

In the last years, great efforts have been implemented in genomics to attempt to understand the genetic determinism of tolerance to environmental stresses, biotic and abiotic, especially in species models (Umezawa et al., 2006; Ashraf, 2010). The same applies to the case of coffee on which the recent progress in genome sequencing resulted in thousands of EST sequences (Lin et al., 2005; Poncet et al., 2006; Vieira et al., 2006; Mondego et al., 2011), for the construction of genetic maps (Lefebvre-Pautigny et al., 2010; LEROY et al., 2011), improvement of genetic transformation techniques (Ribas et al., 2011) and complete genome sequencing of coffee (De Kochko et al., 2010). These scientific advances have paved the way for studies of genetic determinism and molecular drought tolerance in this plant.

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1 REVIEW OF RELATED LITERATURE

1.1 International market

Coffee is the most widely traded tropical agricultural commodity in the world, cultivated around 11 million hectares (ha) in over 80 countries from Africa, Asia, and the Americas. Small stakeholders account for approximately 70% of world coffee production and coffee trade has economic relevance as source of employment for millions of people worldwide.

In 2015/16, the annual world production was around 143.3 million bags of coffee beans (ICO, 2016). The coffee trade statistic showed an increase of 0,7% in global coffee production in 2015/16 crop year compared to 2014/15. Estimated increase in global production of Robusta coffee represented 1.7% in 2015/2016 while no changes were estimated in global production of Arabica coffees in 2015/2016. Over the last four years, Robusta worldwide production increased from 39% to 42,15% as Arabica production decreased in 3,15% (Figure 1).

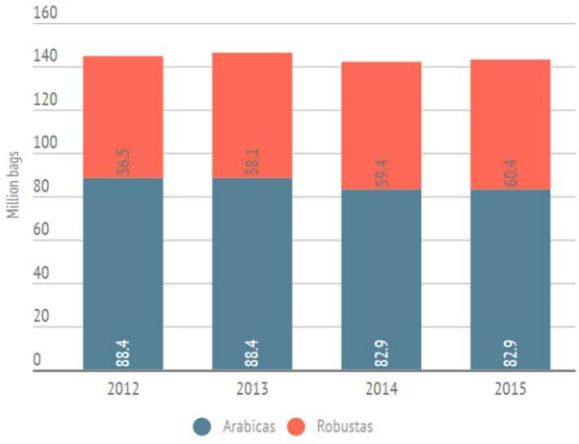

Figure 1 Coffee trade statistic for the last four years of crop production.
Source ICO (2016).

Brazil is the major coffee world producer for more than a century and currently responsible for a third of global production (30,2%), followed by Vietnam (19,2%), Colombia (9,42%), Indonesia (8,59%) and Ethiopia (4,67%) (ICO, 2016). Altogether, these exporting countries contributed around 72% of coffee world production in the 2015/2016 crop year (Table 1).

Table 1 Total coffee production by all exporting countries (in thousands 60 Kg bags) for the last six crop years.

Crop year	2010/11	2011/12	2012/13	2013/14	2014/15	2015/16
Brazil (A/R)	48.095	43.484	50.826	49.152	45.639	43.235
Vietnam (R/A)	20.000	26.500	23.402	27.610	26.500	27.500
Colombia (R/A)	8.523	7.652	9.927	12.124	13.333	13.500
Indonesia (R/A)	9.129	10.644	11.519	11.265	11.418	12.317
Ethiopia (A)	7.500	6.798	6.233	6.527	6.625	6.700
TOTAL	**134.246**	**140.617**	**144.960**	**146.506**	**142.278**	**143.306**

Source ICO (2016).

In 2016 crop year, Brazilian coffee yield is projected at 49,6 million bags and the total area planted is around 1.942,1 thousand ha (CONAB, 2015). Minas Gerais (MG) is the major coffee producer state with 28,5 million bags (57,46%) in which Arabica species represented 67,35% of total coffee area planted in Brazil. On the other hand, Espirito Santo (ES) is the second producer state with 9,5 million bags (19,15%) mainly planted with Robusta (CONAB, 2015).

Arabica trees are forecast to produce 38 million bags in 2015/2016 crop year, up 3.8 million bags compared to the previous season. On the other hand, Robusta production in 2015/16 is expected to decrease to 14.4 million bags, down 2.6 million bags from the previous crop year, especially due to lower agricultural yields in Espirito Santo because of a prolonged dry spell and above average temperatures during the summer months. In addition, Espirito Santo has also faced shortage of water resources, limiting the use of irrigation in coffee plantations which are common in that state (Gain, 2016). Coffee is also growing in other

Brazilian states like São Paulo (10%), Bahia (7,6%), Paraná (2,18%), Rondônia (4,44%) and Goias (6,1%) (CONAB, 2015).

The benefits of coffee consumption are being perceived by consumers and the demand is currently rising. Several epidemiological studies suggest that moderate coffee consumption (3-4 cups/day) may prevent several chronic diseases (Higdon; Frei, 2006) such as diabetes (including type 2 diabetes mellitus) (van Dam; Feskens, 2002; Carlsson, 2004), cardiovascular (coronary heart disease, congestive heart failure, arrhythmias) (O'Keefe et al., 2013), chronic liver illnesses (cirrhosis and hepatocellular carcinoma) (Gallus et al., 2002) and neurodegenerative (Parkinson's, Alzheimer) (Lindsay et al., 2002; van Gelder et al., 2007; Campdelacreu, 2014) ones.

The first bitter mouthful in the morning which gives energy to the planet daily is coffee, one of the most consumed beverages in the world with more than 2.25 billion cups consumed every day. The global coffee consumption was estimated to 149.3 million bags (60kg of green beans) in 2014 (ICO, 2016). Since 2011, coffee consumption averaged annual growth rate of 2.3% (Figure 2).

During the last few years, the demand increased in many countries, particularly in traditional markets (Canada, European Union [EU], Japan, Norway, Switzerland, USA and others), but was also sustained by emerging markets (Algeria, Australia, Russia, South Korea, Turkey, Ukraine, others) and exporting countries (e.g. Brazil) (ICO, 2016). A total of 112.372 thousand bags was imported in 2016, USA being the first in the rank of importing countries with 27.016 thousand of bags (24%). On the other hand, EU imported 72.246 thousand bags (64,2%), among them Germany (18,8%), Italy (7,86%) and France (5,97%) stands out as coffee importers. Japan is closer to Italy with 7,46% of world importations (ICO, 2016).

Figure 2 Global coffee consumption. During last years, the demand increase in many countries including traditional markets, exporting counties and emerging markets.
Source ICO (2016).

Currently, the total domestic consumption by all exporting countries is 47.633 thousand bags (Table 2). Besides Brazil being the main producer, it also leads consumption among exporting countries (42.9%) (Table 2) followed by Indonesia (9,36%) and Ethiopia (7,73%). On the other hand, the European Union stands out (39,82%) the ranking of consumption among importing countries, USA (23,37%) and Japan (7,36%) are in the second and third position, respectively (Table 2). To attend the increasing world consumption of coffee, it is necessary to overcome some challenges in production. Nowadays, drought and high temperatures are the major climatic limitations for world coffee production (DaMatta; Ramalho, 2006).

Table 2 World coffee consumption (in thousand 60Kg bags) for the last four calendar years.

Calendar year	2012	2013	2014	2015
Exporting countries	**44.711**	**455.222**	**46.649**	**47.633**
Brazil	20.178	20.146	20.271	20.458
Indonesia	3.842	4.100	4.292	4.458
Ethiopia	3.387	3.463	3.656	3.681
Importing countries	**98.719**	**102.289**	**103.740**	**104.572**
European Union	41.018	41.875	42.215	41.638
USA	22.232	23.417	23.767	24.441
Japan	7.131	7.435	7.494	7.695
TOTAL	**143.430**	**147.811**	**150.389**	**152.204**

Source ICO (2016).

These abiotic stresses are expected to become increasingly important in several coffee growing regions due to the recognized changes in global climate and also because coffee cultivation has spread towards marginal lands, where water shortage and unfavorable temperatures constitute major constraints to coffee yield.

1.2 Global Climate Change: impacts in coffee production

1.2.1 Impacts in coffee areas

Global climate change is becoming more unpredictable and abiotic stresses are the major cause of decreasing the average yield of principal crop species (Hazarika et al., 2013). Climate changes is occurring at rates never experienced before by modern agriculture, with temperatures planned to increase of 2-3°C over the next 40 years (Hatfield, 2013). This will affect all not only growth and development of plants, but also the quality of their products. When evaluating the effects of climate changes on plants, it is important to include the

direct effects of perennial plants because adaptation strategies for these production systems are more complex than in annual crops.

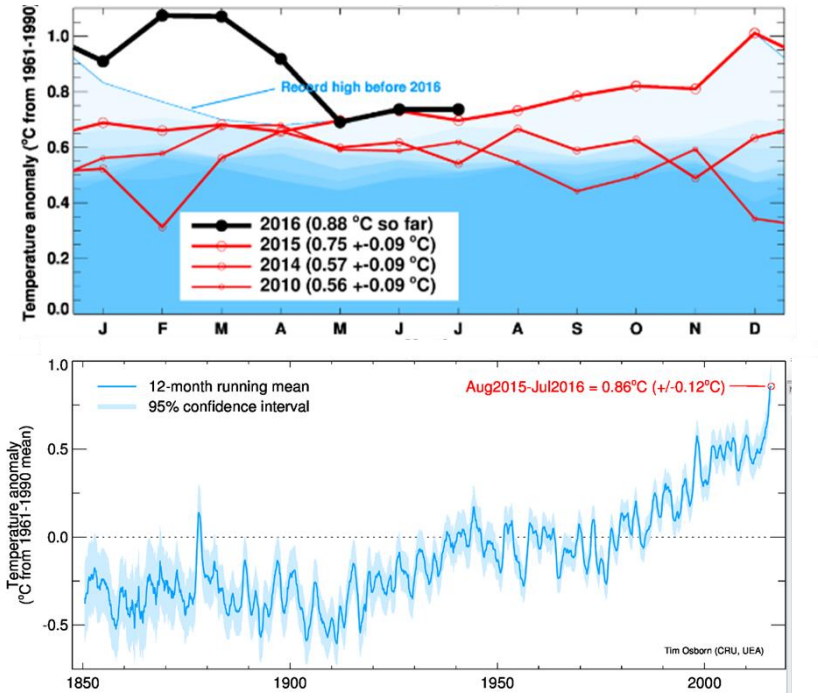

Figure 3 Global-mean temperature anomalies for the current year. The red lines show the monthly temperature anomalies for the 3 warmest years. The blue line near the top shows the record high for each individual month prior to the current year. The bottom graph shows series and 12-month running means values yearly global temperature graphs anomaly time series 1850-2010.

Source Available at https://crudata.uea.ac.uk/~timo/diag/tempdiag.htm - Morice et al., (2012).

As a consequence of global warming, coffee-growing geographical regions could also suffer important geographical delocalization (Assad et al., 2004). In marginal regions without irrigation or during dry seasons, this could led in decreasing coffee yields as much as 80% (DaMatta; Ramalho, 2006). Analyzing the effects of recent climate change by extrapolating the historical tendencies in temperature and precipitation to 2020 in coffee producing areas in

Veracruz, Mexico, the analysis predict that coffee production is likely to decline about 34%. The suitability for coffee crops in Costa Rica, Nicaragua and El Salvador will be reduced by more than 40% (Glenn et al., 2014) while the loss of climatic niches in Colombia will force the migration of coffee crops towards higher altitudes by mid-century (Ramirez-Villegas et al., 2012).

In Brazil, it is expected that coffee areas will migrate towards more favorable zones in the South of country under future climate change (Assad et al., 2004). Some studies have mapped the changes in area suitable for coffee production in the four main coffee producing states as a consequence for global warming (Assad et al., 2004; Pinto et al. 2007). According to the last report of the Intergovernmental Panel on Climate Change (IPCC, 2014), an increase of 3º C in temperature would lead to major changes in the distribution of coffee producing zones. In the main coffee producing states of Minas Gerais and São Paulo, the potential area for production would decline from 70-75% of the states to 20-25%, while coffee area would be reduced by 10% in Paraná and production would be eliminated in Goias state (Figure 4). The new areas suitable for coffee production that could emerge in Santa Catarina and Rio Grande do Sul will only partially compensate the loss of area in other states (Pinto; Assad, 2008).

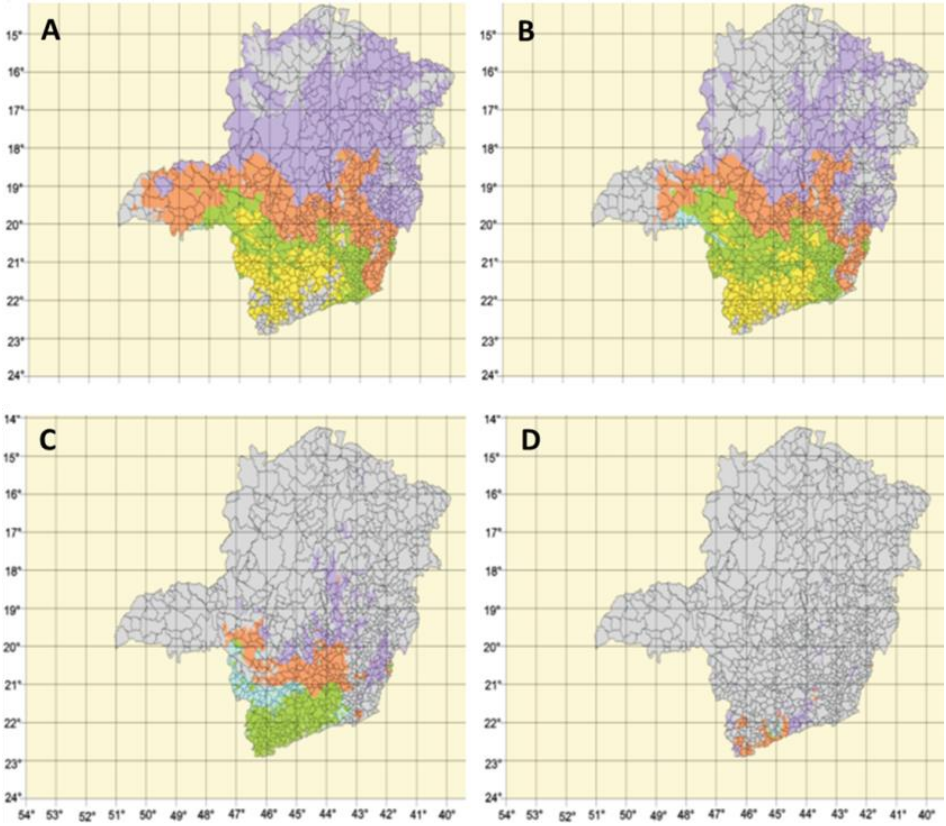

Figure 4 Current coffee zoning for Minas Gerais state. Minas Gerais state with the increase of 1°C in temperature and 15% in rainfall (A); Considering 3°C rise in temperature and 15% in rainfall (B); With the increase of 5,8°C in temperature and 15% in rainfall (C). The colored regions indicate: irrigation required (purple); suitable for cultivation (green); irrigation recommended (orange); frost risk (yellow); thermal excess (light blue); unsuitable for cultivation (gray).
Source Adapted from Assad et al., (2004).

These forthcoming scenarios require new approaches that develop innovative strategies to manage the crop production system and reduce the impact of climate change. Strategies such as the developing agroforestry production systems, increase irrigation, and modify agricultural practices maintaining cover crops are projected to become more frequent.

1.2.2 Impact in term of abiotic stress

Under drought and high temperatures, some coffee pests and diseases should also become more severe. The occurrence of leaf miners (*Leucoptera coffeella*) disease has been increasing over recent years in coffee yields as a consequence of dry conditions (Assis et al., 2012). On the other hand, leaf rust disease is rising with warmer temperatures. Likewise, the number of cycle life generations of *Hyphotenemus hampei* has been increasing under the same climatic conditions, as a result, a thermal tolerance of the coffee berry borer has been demonstrated (Jaramillo et al., 2009). In the case of *Leucoptera coffeella* and *Meloidogyne incognita*, the same circumstances have been predicted in Brazil under these climate change conditions. Therefore, the coffee production demands nowadays plants better adapted to both abiotic and biotic stresses.

1.2.3 Impact on coffee plants

Long periods of drought can beget diverse effects on coffee plants. Moderate drought can promote leaf falling, delay and un-synchronize flowering, reduce vegetative growth of plagiotropic branches and consequently production potential in following crop year, upon severe drought yet major effects are expected up to plant death, abortion of flowering and fruits.

Besides the loss of coffee production and changes in distribution of coffee producing zones, the biochemical composition of beans could also be modified by drought. Variations in rainfall and temperatures affect sugar, proteins and caffeine contents (Mazzafera, 2007) and consequently the beverage quality (Camargo et al., 1992; Vinecky et al., 2016). Moreover, the predicted climate change and the increasing world population will lead to a growing demand for water and reveal the urgent need for drought tolerant crops (Alter et al., 2015).

Nowadays, coffee production demands plants better adapted to both abiotic and biotic stresses. In such way, it is worth noting that the drought-tolerant (D^T) clone 14 of *C. canephora* (Marraccini et al., 2012) was also recently reported to present durable multiple resistant plant to root-knot nematodes of *Meloidogyne* spp. (Lima et al., 2015).

1.3 *Coffea* genus

The *Coffea* genus belongs to Rubiaceae family, the fourth largest flowering plant family in the world, consisting of more than 11.000 thousand species in 660 genera (Robbrecht; Manen, 2006) which represent 10 to 20% of the total plant species diversity. The most economically valuable genus is *Coffea* that contains 124 species which comprises perennial species all native from Madagascar, Africa, the Mascarene Island, the Comoros Island, Asia and Australia (Davis et al., 2006, 2012).

Among all species, *C. arabica* and *C. canephora* are the two economically important species corresponding to 65% and 35% of the international market, respectively (ICO, 2016). The two species are perennial woody trees and display considerable variation in morphology, size, and ecological adaptation (Combes et al., 2015). Nevertheless, *C. arabica* is an allotetraploid (2n = 4x = 44) that was originated 1 million years from the natural hybridization of two ancestral diploid genomes, *C. canephora* and *C. eugenioides* (Figure 5). As provider of a higher quality beverage *C. arabica* is the most cultivated specie (Poncet et al., 2007).

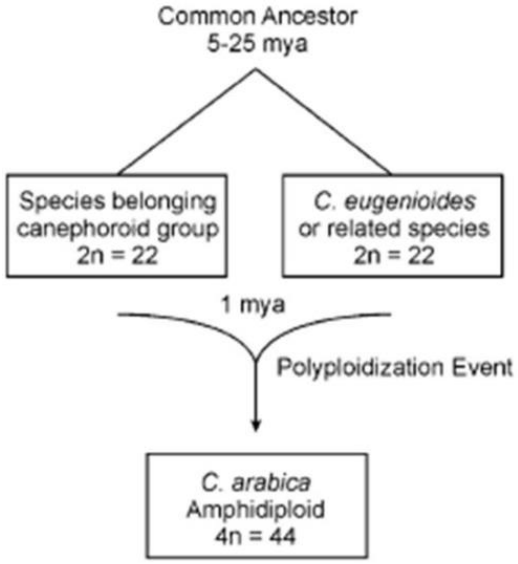

Figure 5 Evolutionary history of allotetraploid *C. arabica*. The progenitor genomes are represented by diploid *C. eugenioides* and *C. canephora*. *C. arabica* arose 1 to 2 million years ago (mya) from the fusion of *C. canephora* (or related species) and *C. eugenioides*.
Source Vidal et al., (2010).

1.3.1 *Coffea arabica*

Originally from Southwest Ethiopia and Plateau of Sudan, *C. arabica* was cultivated about 1,500 years ago, firstly in Ethiopia. The genetic background of the current *C. arabica* cultivars comes from Typica and Bourbon (Anthony et al., 2002). As a predominant autogamous (natural self-pollinating) specie, *C. arabica* present low genetic diversity (Hatanaka et al., 1999) and has a total genome size estimated by flow cytometry at around 2.62 x 10^3 Mb (Clarindo; Carvalho, 2009). The breeding programs nowadays have been search new cultivars with improved traits such as beverage cup quality, flowering time synchronicity, resistance to pests, and drought stress tolerance.

As *C. arabica* is an amphidiploid species (originating from a natural hybridization event between *C. canephora* and *C. eugenioides*), its transcriptome is a mixture of homologous genes expressed from these two subgenomes in which *C. eugenioides* is assumed to expressed genes mainly for proteins involved in basal biological process as photosynthesis, while *C. canephora* sub-genome is assumed to regulate Arabica gene expression by expressing genes for regulatory proteins and adaptation process (Vidal et al., 2010).

1.3.2 *Coffea canephora*

C. canephora is a cross-pollinated diploid species (2n = 2x = 22) that has high genetic variability in its haploid genome of 710 Mb (Denoeud et al., 2014). Thereby, exist genetic variability within the *Coffea* genus that could be used to increase drought tolerance and among commercial species *C. canephora* stands out. Despite the ability of *C. canephora* to adapt regarding various climatic conditions (Bertrand et al., 2003), it produces beans giving lower quality beverage that are more used in instant coffee drinks (Hatanaka et al., 1999).

C. canephora genetic diversity can be divided in two major clades according to their geographical origins: the Guinean group (G) and the Congolese group. The Congolese group can be subdivided into SG2/B, C, SG1 and UW (Montagnon; Leroy, 1993) (Figure 6). Guinean genotypes are considered the most tolerant to drought and genotypes from the SG1 Congolese group are more tolerant to drought than those from the SG2 Congolese group (Montagnon; Leroy, 1993). The considerable genetic diversity observed in *C. canephora* is still largely unexploited. During the last decade, several breeding programs to development of new *C. canephora* clones have attempted to explore the genetic diversity of *C. canephora.* In Brazil, a genetic improvement program for the development of new cultivars, using SG1 genotypes as source of genetic variability, characterized a

clonal variety of *C. canephora* Conilon highly productive under drought conditions (Ferrão et al., 2000).

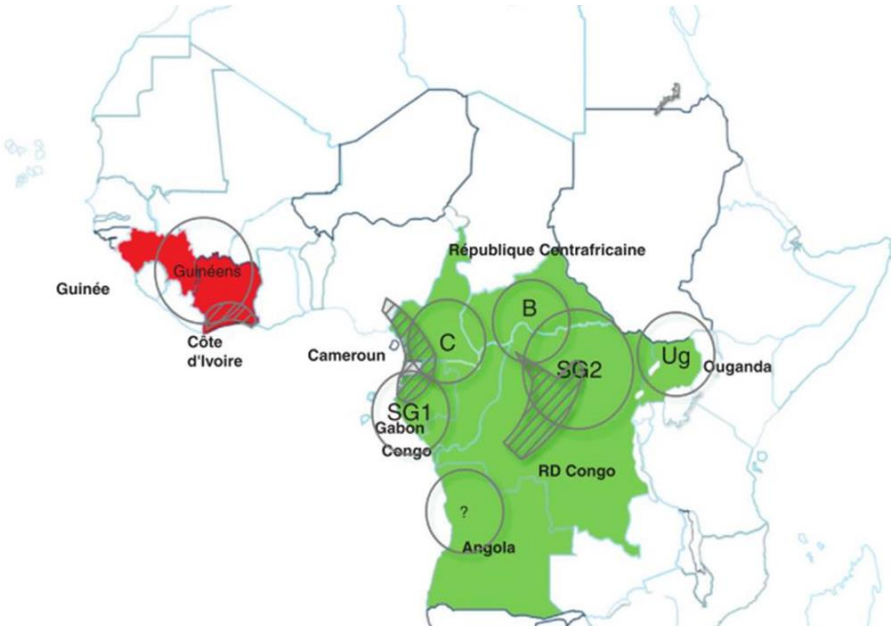

Figure 6 Geographic origin of the two main genetic group of *C. canephora*. In red: geographic origin of the Guinean group. In green: geographic origin of the Congolese subgroups (SG). The circles highlight the identification of each subgroup.
Source Montagnon et al., (2012).

1.3.3 Other *Coffea* species

Even though *Coffea* genus diverged recently (5 to 25 million years ago) from others plants, most of their species are genetically highly related thus permitting natural or manual hybridizations that could be used in coffee breeding programs. For instance, it has been introduced in *C. arabica* by breeding programs resistance genes for leaf rust (*Hemileia vastatrix*), for the *Meloidogyne* nematodes, and to *Colletotrichum kahawae* fungus agent of Coffee Berry Disease (CDB) (Bertrand et al., 2003).

In this sense, the diploid species *C. racemosa* presents high resistance to drought and elevated temperatures. In its native habitat, *C. racemosa* is able to adapt to regions where the annual rainfall does not exceed 1000 mm and where dry seasons vary from four to six months (Krug, 1965; Dublin, 1968). This specie presents deeper growth of primary root and lower growth of secondary roots allowing this specie to explore deeper soil layers in water deficit conditions (Fazuoli, 1975). *C. racemosa* had the longest root system in comparative analyses with other coffee species (*C. canephora, C. arabica, C. liberica and C. congensis)* and the root system is mainly contrasting with *C. congensis* root system which survived in a natural environment completely different of *C. racemosa* (Dublin, 1968).

Medina Filho et al. (1977b) had evaluated the genetic material of *C. racemosa* from Campinas (Brazil), and they verify that triploids (*C. arabica* x *C. racemosa*) as well as individuals belonging to the second generation backcrosses to *C. arabica* were highly resistant to drought, while Catuai and Acaia cultivars of *C. arabica* (positive controls of the experiment), were highly sensitive. While these cultivars lose a lot of leaves the plants which derivate of *C. racemosa* keep their leaves notably turgid.

1.4 Drought responses in plants

Drought is one of the major constraints of plant productivity worldwide. Under field conditions, plant performance in terms of growth, development, biomass accumulation and yield depends on acclimation ability to the environmental changes and stresses, exercising specific tolerance mechanisms that involve a complex network of biochemical and molecular processes (Wang et al., 2003). When exposed to reduce water availability plants exhibit various physiological responses. For instance, a pivotal reaction is stomatal closure to avoid water loss by transpiration. The resulting reduced availability of carbon

dioxide together with a down regulation of photosynthesis-related genes lead to decrease in carbon assimilation restricting plant growth and productivity (Alter et al., 2015). Under drought stress conditions, an increase in photorespiration leads to an accumulation of reactive oxygen species (ROS), which are toxic for cellular components and will eventually lead to cell death (Mittler, 2002). Plants have evolved a number of molecular and physiological adaptation mechanisms to cope with reduced water availability which can be categorized into drought avoidance and drought tolerance (Verslues et al., 2006) (Figure 7).

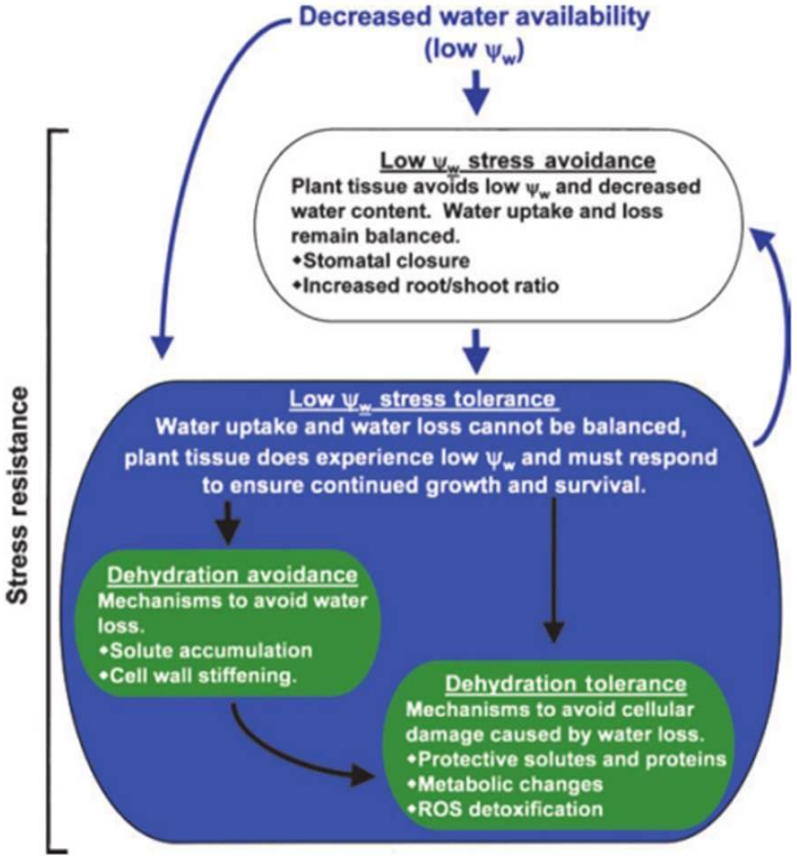

Figure 7 Conceptual diagram of the stress tolerance/stress avoidance model of low $-\Psi_w$ responses.
Source Verslues et al., (2006).

In most cases, the plant first response is avoid low Ψ_w. Tissue Ψ_w and water content are maintained close to the unstressed level by increasing water uptake or limiting water loss by such that the rates of water loss and water uptake remain balanced. Such a balance is achieved in the short term mainly by stomatal closure. In long term, changes in root and shoot growth, leading to an increased root/shoot ratio, tissue water storage capacity and cuticle thickness and water permeability are also of potential importance. Of these, changes in root growth to maximize water uptake are of the greatest importance for crop plants (Verslues et al., 2006).

Furthermore, these mechanisms for avoiding water loss do not themselves offer any protection from the effects of low Ψ_w if the stress becomes more severe and the plant is no longer able to maintain a balance between water uptake and loss. When stomata are closed because of stress, transpiration is minimized, the Ψ_w of the plant will equilibrate with that the water source (most of cases Ψ_w of the soil). When soil water content and Ψ_w are low, the Ψ_w of the plant tissue must also decrease, either through water loss or by adjustment made by the plant (dehydration avoidance) to achieve a low Ψ_w while avoiding a water loss. The main mechanism of dehydration avoidance are accumulation of solutes and cell wall hardening (Verslues et al., 2006).

The Ψ_w of a walled cell, such as a plant cell, is governed by the equation: $\Psi_w = \Psi_s + \Psi_p$, where Ψ_s is the osmotic potential and Ψ_p is the pressure potential (turgor pressure). At a given Ψ_w, a higher Ψ_p can be achieved by accumulating solutes inside the cell, thus lowering Ψ_s. The accumulation of additional solutes in response to low Ψ_w is termed osmotic adjustment (Zhang et al., 1999). Osmotic adjustment refers to the active accumulation of additional solutes in response to low Ψ_w (after the effect of reduced water content on the concentration of existing solutes has been factored out). Thus, many plants accumulate one or more types of compatible solutes, such as proline or glycine betaine, in response to low Ψ_w

(Verslues et al., 2006). Compatible solutes can also protect protein and membrane structure under dehydration (Hincha; Hagemann, 2004).

In this way, a key regulatory which control plant responses to many types of abiotic stress (including low Ψ_w) is the phytohormone abscisic acid (ABA). It accumulates in response to abiotic stress and regulates the processes involved several the aspects of the low-Ψ_w response. For instance, ABA-regulated stomatal conductance, root growth and seed dormancy (Schroeder, et al. 2001; Sharp; LeNoble, 2002; Kermode, 2005) which are important in avoidance of low Ψ_w. Moreover, ABA induces accumulation of compatible solutes which can be crucial for dehydration avoidance (Ober; Sharp, 1994) and ABA also regulates dehydrins and LEA proteins synthesis, important for dehydration tolerance (Sivamani et al., 2000). Thus, at the level of the organism, it seems that a main function of ABA is to coordinate the various aspects of low-Ψ_w response.

1.4.1 Coffee genetic diversity and drought

Among the strategies displayed by coffee plants to cope with drought, leaf folding and inclination that reduce the leaf surface (Figure 8), water loss by transpiration and exposure to high irradiance were commonly observed for Guinean and SG1 genotypes (Montagnon; Leroy, 1993). Leaf abscission is then reduced, favoring a rapid recovery of vegetation with the return of the rains. Such a trait can be considered as a selective advantage when compared with the leaf abscission that characterizes SG2 genotypes (Marraccini et al., 2012).

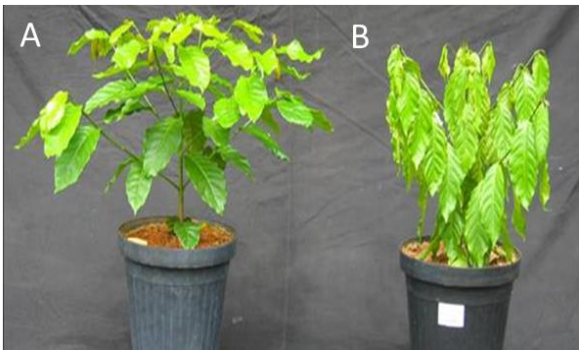

Figure 8 *C. canephora* clones (A: Drought tolerant, D^T; B: Drought susceptible, D^S) grown in greenhouse and submitted to drought conditions.
Source From author (2016)

Several drought-tolerant clones (D^T) of *C. canephora* var. Conilon have been characterized as vigorous plants with high productivity throughout years under drought stress (Ferrão, 2000; Fonseca, 2004). Fingerprint analyses also revealed that these Conilon clones belong to the SG1 group of *C. canephora* (Lambot et al., 2008; Montagnon et al., 2012).

Regarding *Coffea arabica*, the study of populations from Ethiopia growing under contrasting climatic conditions also revealed that this species exhibited phenotypic plasticity in response to varying soil moisture conditions (Beining et al., 2008). It is well known that a genetic variability for drought tolerance also exists in *C. arabica*. For instance, the cultivar IAPAR59 (I59), which the result of a cross between the Timor hybrid HT832/2 and the Villa Sarchi cultivar is considered more tolerant to drought than the Rubi cultivar that did not undergo recent introgression with *C. canephora* genomic DNA (Marraccini et al., 2001; Mofatto et al., 2016).

Figure 9 Contrasting phenotypes of the drought-tolerant I59 (A) and drought-susceptible Rubi (B) cultivars of *C. arabica* in response to a drought period of around 200 days without rainfalls (Embrapa Cerrados).
Source From author (2016)

Major differences between these two cultivars concerned their phenotypic behavior (Figure 9) as predawn leaf water potential, Ψ_{pd} (Figure 10) and transcriptome expression profiles. Marraccini et al. (2011) evaluated the effect of drought in leaves of young plantas of *C. arabica* cv. I59 and Rubi cultivars grown in field with irrigation (I) or without (NI) irrigation during two consecutive years (2008 and 2009). As result, the Ψ_{pd} values measured during the dry season of 2008 and 2009 were almost less negative for the D^T I59 than for D^S Rubi, indicating a better access to soil water for the former compared to the latter (Marraccini et al., 2011).

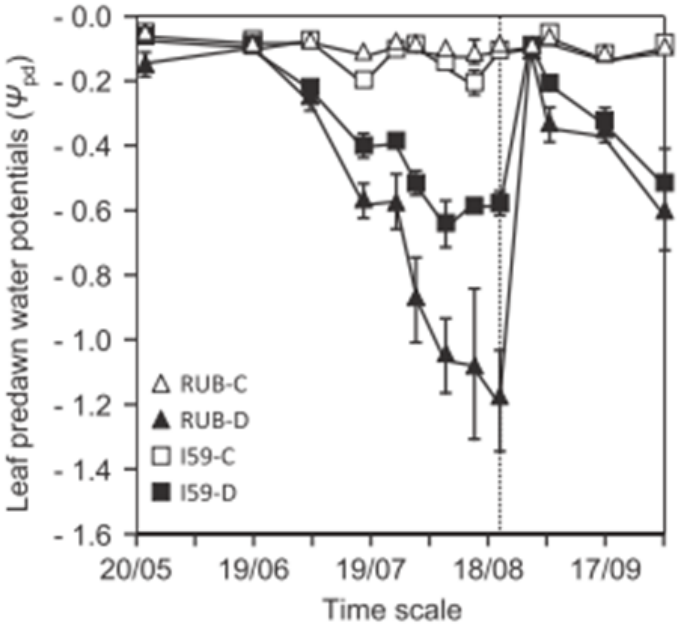

Figure 10 Predawn leaf water potential (Ψ_{pd}) measured in *C. arabica* plants. *C. arabica*, Rubi (RUB, triangle) and IAPAR (I59, square) cultivars were grown under control (C, open symbols) and drought (D, black symbols) conditions. Ψ_{pd} values (expressed in mega-Pascal, MPa) were measured once a week during the 2009 dry season (23-month-old plants).
Source Mofatto et al., (2016).

1.4.2 Physiological responses

The Kouillou (SG1) group of *C. canephora* appears to be more tolerant to water deficit than Robusta (SG2) (Montagnon; Leroy, 1993). SG1 group maintain stomatal opening and consequently active photosynthesis, while stomata of SG2 plants were completely closed under drought conditions.

Besides that, more efficient root water absorption for the SG1 plants could explain its drought tolerance albeit its maintenance of stomatal opening (Boyer, 1969). Physiological analyses also suggested that drought tolerance could be a direct consequence of better root development (Pinheiro et al., 2005) (Figure 11).

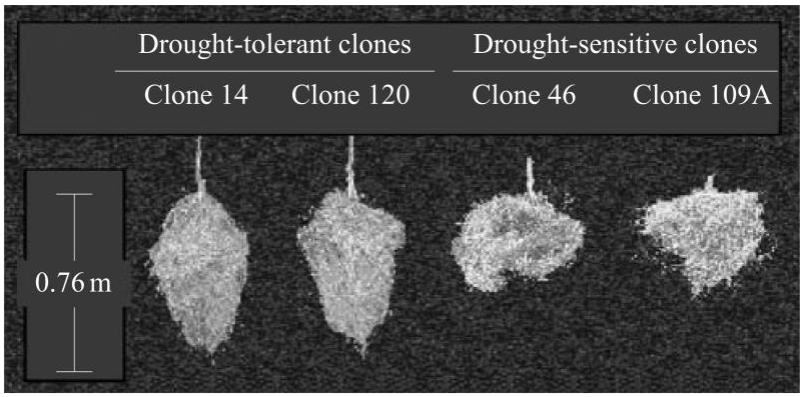

Figure 11 Typical root systems of four clones of Robusta coffee grown under full irrigation.
Source Pinheiro et al., (2005).

One of the physiological parameters that distinguish the drought-susceptible (D^S) clone 22 of *C. canephora* var. Conilon from the D^T clones 14, 73 and 120 is the rate of decrease in the predawn leaf water potential (Ψ_{pd}) (RDPW) (Pinheiro et al., 2004). To reach the imposed Ψ_{pd} of -3.0 MPa under the stressed (NI) conditions in the greenhouse, the RDPWP decrease faster for the D^S clone 22 than for the D^T clones (Figure 12). In this condition, the clone D^S 22 reached the Ψ_{pd} of -3.0 MPa within six days, while clones 14, 73 and 120 reached the same within 12, 15 and 12 days, respectively (Marraccini et al., 2011).

According to DaMatta et al. (2003), the better crop yield of a drought-tolerant clone compared with a drought-sensitive clone is mainly associated with the maintenance of leaf area and tissue water potential that are consequences of reduced stomatal conductance (g_s). The D^T and D^S clones of *C. canephora* are important models of study once a lot of physiological and molecular parameters were already evaluated in these plants concerning drought stress under controlled conditions. It is worth noting that the drought-tolerant (D^T) clone 14 of *C. canephora* (Marraccini et al., 2012) was also recently reported to present durable

multiple resistant plant to root-knot nematodes of *Meloidogyne* spp. (Lima et al., 2015).

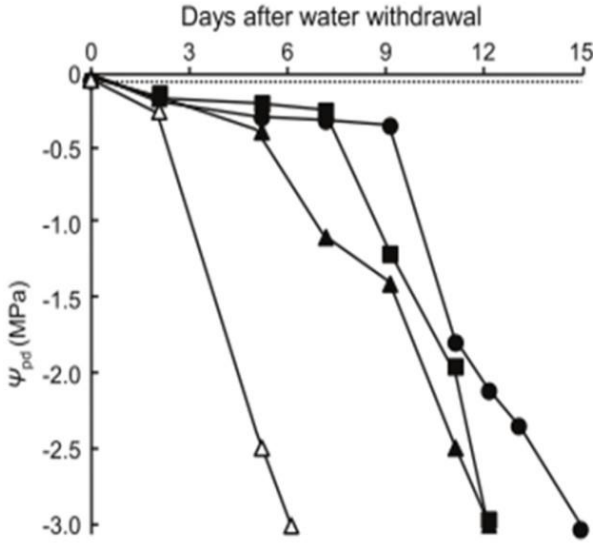

Figure 12 The evolution of predawn leaf water potential (Ψ_{pd}) in the leaves of *C. canephora*. The clones 14, 22, 73 and 120 of *C. canephora* var. Conilon were grown in a greenhouse under water stress. For each clone, Ψ_{pd} evolutions are presented.
Source Marraccini et al., (2011).

1.4.3 Biochemical responses

The activity of antioxidant enzymes might also be involved in the drought tolerance mechanism (Vieira et al., 2006). A key role of ascorbate peroxidase (APX) was postulated to allow clone 14 to cope with potential increases of H_2O_2 under drought conditions, as an increased (38%) activity of this enzyme was found for this clone upon drought stress (Pinheiro et al., 2004). Praxedes et al. (2005) observed a maintenance of SPS activity with the decrease of pre-dawn leaf water potential (Ψ_{pd}) for the drought-tolerant clone 120 but not for the drought-sensitive clones.

1.4.4 Molecular responses

Several differentially expressed genes and proteins were investigated in leaves of drought-tolerant and susceptible *C. canephora* clones upon drought acclimation. Genes coding for protein functioning as secondary messengers (*CcNSH1*, *CcEDR1* and *CcEDR2*), related to abscisic acid (ABA) perception and signal transduction (*CcPYL3*, *CcPYL7* and *CcPP2C*), transcription factors (*CcABI5*, *CcAREB1*, *CcRD26*, *CcDREB1*), photosynthesis (*CcPSBP*, *CcPSBQ*, *CcRBCS1*), and drought protection (*CcHSP1*, *CcDH3*, *CcAPX1*), were previously characterized (Marraccini et al., 2012; Vieira et al., 2013).

Recently, among the 42 genes showing up-regulated expression in plagiotropic buds of plants submitted to drought were *CaSTK1* (coding a protein kinase), *CaSAMT1* (coding a protein involved in abscisic acid biosynthesis), *CaSLP1* (coding a protein involved in plant development) and several "no-hit" (orphan) genes of unknown function. Under water scarcity, the expression of *nsLTPs* (coding non-specific lipid-transfer proteins) was greatly up-regulated specifically in plagiotropic buds of I59 which could explain the thicker cuticle observed on the abaxial leaf surface in the D^T I59 compared with the D^S Rubi (Mofatto et al., 2016).

All this information could be used to generate molecular markers to be used in *Coffea* breeding programs for both *C. arabica* and *C. canephora* plant. In this context, 436 plants of *C. canephora* (LxPy) were selected among a population of 3500 individuals from 48 progenitors based on traits of interest such as precociousness of fruit, plant vigor, productivity in field (Carneiro et al., 2015). These plants grown in field conditions since 2009/2010 were submitted to drought conditions and evaluated for their productivity and Ψ_{pd} under drought (winter) season (Figure 13). This allowed the identification of productive and drought-

tolerant plants (e.g. L13P63, L8P68 and L5P47) that contrasted with drought-susceptible and lower productive plants (L12P57, L12P100 and L15P14).

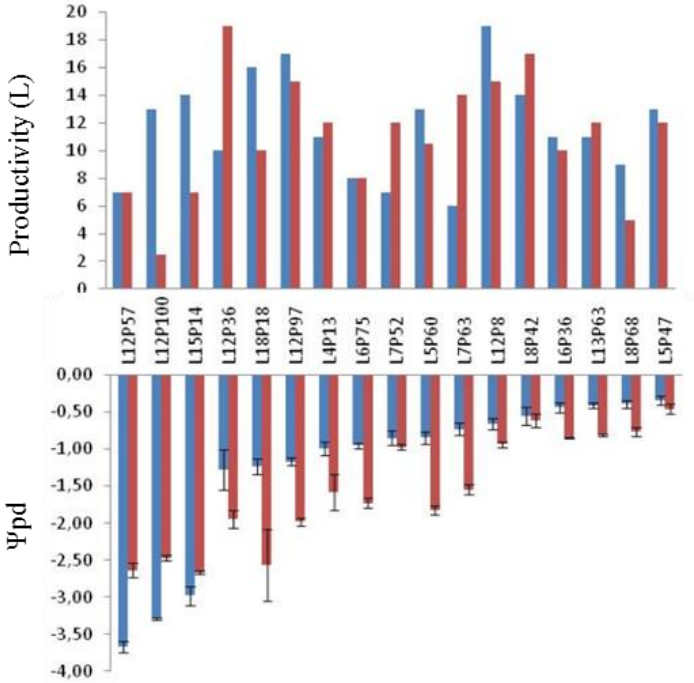

Figure 13 The productivity (measured in liters of cherries per plant) and Ψ_{pd} of LxPy plants of *C. canephora* Conilon grown in field conditions (Embrapa Cerrados) under drought stress. These values were measured during two years (2009: blue isobars and 2010: red isobars).
Source Carneiro et al., (2015).

1.5 ABA structure and biological roles

The abscisic acid (ABA), discovered in the 1960's (Ohkuma et al., 1963; Cornforth et al., 1965) is a vital hormone synthesized mainly in leaves and roots of the plants (Zhang; Davies, 1989; Thompson et al., 2007), acting as central regulator that protects plants against abiotic stresses such as drought (Wasilewska et al., 2008; Soon et al., 2012). This sesquiterpenoid molecule ($C_{15}H_{20}O_4$)

naturally occur in its S-(+)-ABA form, despite the R-(-)-ABA form is active in some assays (Cutler et al., 2010) (Figure 14).

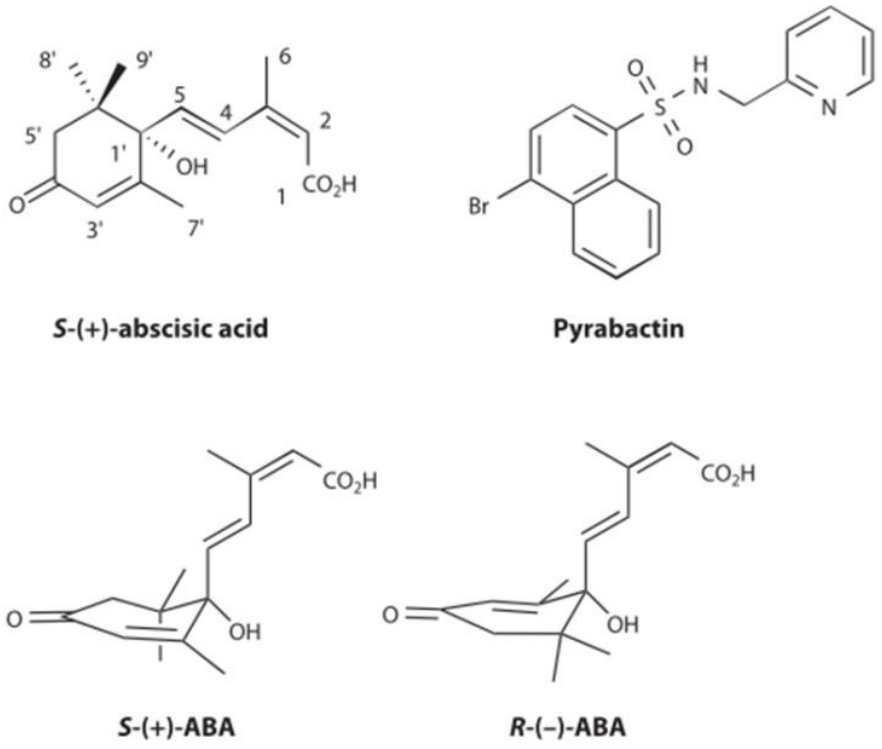

Figure 14 Chemical structures of ABA. At bottom is an illustration of the ability of an abscisic acid (ABA) stereoisomer to be rotated along its lengthwise plane to maintain positioning of polar functional groups.
Source Cutler et al., (2010).

ABA has been shown to control many aspects of plant growth and development as embryo maturation, seed dormancy, germination, cell division and elongation and floral induction (Finkelstein, 2013). ABA is well known as 'stress hormone' and it plays a key role not only during drought (Santiago et al., 2009a; Gonzalez-Guzman et al., 2014) but under other abiotic stresses such as salinity (Pons et al., 2013), cold (Bhyan et al., 2012; Shinkawa et al., 2013) and UV radiation (Tossi et al., 2012; Chen et al., 2012). Moreover, ABA has an

important function as well in biotic stresses acting in plant immunity (Adie et al., 2007; Fan et al., 2009; Robert-Seilaniantz et al., 2011; Ramegowda; Senthil-Kumar, 2015).

1.5.1 ABA biosynthesis, catabolism, conjugation and transport

The increase of ABA levels in the leaves and roots after drought stress was very limited in the ABA-deficient *Arabidopsis* mutant *aao3-1,* which has a defect in a final step of ABA biosynthesis, indicating that the increase in ABA levels after stress treatment is due to the activation of de novo ABA biosynthesis (Ikegami et al., 2009). ABA can also be rapidly release from cellular stores of conjugated glycosyl ester form by glucanases activated or stabilized by dehydrating stress (Lee et al., 2006; Xu et al., 2012)

Similarly the most plant hormones, ABA levels reflect a balance of ABA biosynthesis and inactivation by turnover or conjugation, further modified by compartimentation and transport (Figure 15). In plants, ABA is synthesize from carotenoids and it is known to be transported over long distances (Jiang; Hartung, 2008). As a weak acid, ABA is mostly uncharged when present in the relatively acid apoplastic compartiment of plants and analyses uptake does not occur solely by a diffusive process since active ABA transporters were also reported to participate to its uptake (Jiang; Joyce, 2003). Among multiple plasma membrane-localization transporters that have been recently identified, two ATP-binding cassete (ABC) transporters were identified as an importer (AtABCG40) and exporter (AtABCG25) of ABA, and genetic analyses demonstrated their importance for ABA responses including stomatal regulation, gene regulation, germination inhibition and stress tolerance (Kang et al., 2010; Kuromori et al., 2010).

The site of stress perception and that of ABA biosynthesis during the drought stress have been extensively discussed (Sauter et al., 2001; Ikegami et al.,

2009; Hartung, 2002; Jeschke et al., 1997). There are evidences that shoot transpiration rate is largely dependent of the delivery of ABA from the roots and the sensitivity to ABA in response to water deficit. In this context, roots are able to 'measure' decreasing soil water availability during a period of drought which results in an increased release of ABA from the roots tissues to the xylem vessels. After xylem transport to the shoot, guard cells respond rapidly and sensitively to increased ABA concentrations resulting in reduced transpirational water loss (Sauter et al., 2001). Some of the ABA synthesised in the dry roots may be transported to the shoot through the xylem with the transpiration stream and accumulate in high levels in the leaves (Hartung, 2002). Under conditions of soil drying and salt stress large amounts of ABA are deposited in root tissues and loaded into the xylem. Sometimes ABA synthesis by roots is increased substantially but root ABA concentrations may not increase because most of this newly synthesized ABA is loaded to the xylem and transported to the leaves (Jeschke et al., 1997). It could also occur once ABA may move freely from plant to soil and to soil from plant (Sauter et al., 2001).

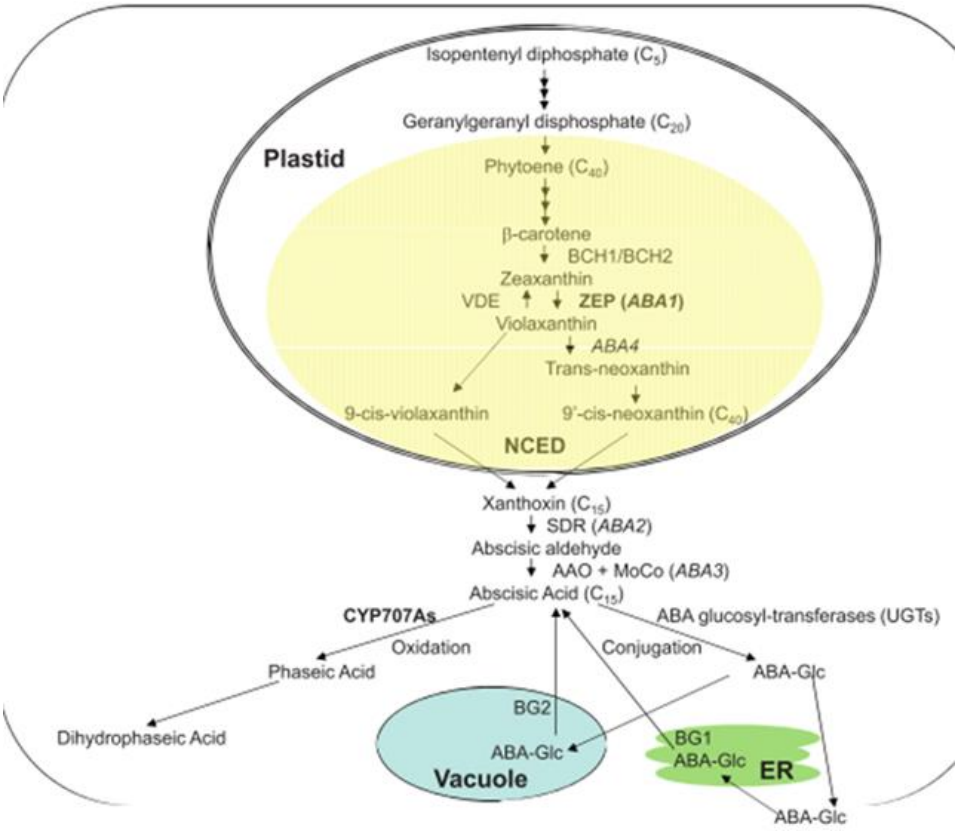

Figure 15 ABA metabolic pathways. ABA biosynthesis, degradation and conjugation pathways are shown in relation to the cellular compartments where these events occur. Carotenoid intermediates are highlighted in yellow. Enzymes regulating key regulatory steps are shown in bold. Individual loci identified based on ABA deficiency are shown in italics.
Source Finkelstein (2013)

On the other hand, it have been demonstrated also that ABA is synthesized mainly in the leaves in response to drought stress and that some of the ABA accumulated in the leaves is transported to the roots (Ikegami et al., 2009). In this work, tracer experiments using isotopelabeled ABA indicate that the movement of ABA from leaves to roots is activated by water deficit in roots (Ikegami et al., 2009). When roots were kept in well-watered conditions and

drought stress was localized to the leaves only, the ABA level in the leaves increased as in the case of intact plants and detached leaves. Further, under these conditions, the ABA level in the roots did not differ from that in the well-watered control. On the other hand, when drought stress was localized to the roots only, the ABA level in the leaves was slightly higher than that in the well-watered control. Consistent with the ABA levels, leaf stomata closure was almost complete after localized stress treatment to leaves, and was partially induced when drought stress was localized to roots only (Ikegami et al., 2009).

The role of ABA in controlling plant responses likely involves actions at several levels, including effects on transcription, RNA processing, post-translational protein modifications, and the metabolism of secondary messengers (Figure 16). Almost 200 loci regulating ABA response and thousands of genes are regulated by ABA under different contexts (Finkelstein, 2013).

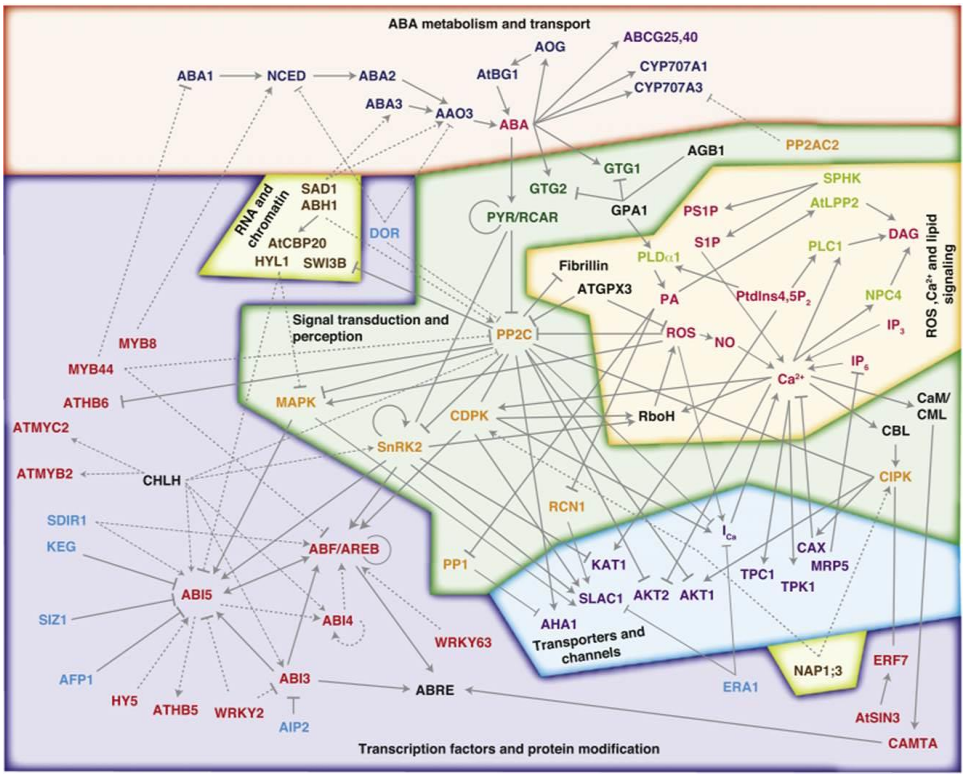

Figure 16 The ABA signaling network. The network is divided into six main functional categories: ABA metabolism and transport (red); perception and signal transduction (dark green); ROS, Ca^{2+} and lipid signaling (orange); transporters and channels (blue); transcription factors and protein modification (purple); and RNA processing and chromatin remodeling (light green).
Source Hauser et al., (2011).

1.6 The PYL/PP2C/SnRK2: the first steps of ABA sensing and signaling

Over the past few decades, a lot of work was done elucidating the molecular mechanisms underlying ABA sensing and signaling (Umezawa et al., 2010). Several putative ABA receptors, including FCA (Razem et al., 2006), CHLH (Shen et al., 2006), GCR2 (Liu et al., 2007), GTG1 and GTG2 (Pandey et al., 2009) were reported to bind ABA with varying affinities. The discovery of PYLs candidate ABA receptors was different from that of the earlier putative

ABA receptors, once independent findings from several groups converged upon this novel class of ABA binding proteins, which fit elegantly into a model that connected the core components of the ABA signal transduction pathway (Ng et al., 2014).

The tripartite ABA signaling pathway is initiated by ABA perception through the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) family of proteins (Ma et al., 2009; Park et al., 2009). These novel intracellular ABA receptors (PYL/RCARs) are involved in ABA sensing and signaling via their direct interaction with clade A protein phosphatase type 2C (PP2Cs), such as ABA INSENSITIVE1 (ABI1) and ABI2, HYPERSENSITIVE TO ABA1 (HAB1) and HAB2, and PROTEIN PHOSPHATASE 2CA/ABA-HYPERSENSITIVE GERMINATION3 (PP2CA/AHG3), thereby releasing their inhibition on three ABA-activated SNF1-related protein kinases (SnRK2s), SnRK2.2/D, 2.3/I and 2.6/E/OST1 (Umezawa et al., 2009; Vlad et al., 2009).

The current ABA signal transduction model can be described as follow: in the absence of ABA, SnRK2 kinases are inactivated by PP2Cs which physically interact with SnRK2 and dephosphorylate a serine residue in the kinase activation loop, a phosphorylation essential for kinase activity (Belin et al., 2006) (Figure 17).

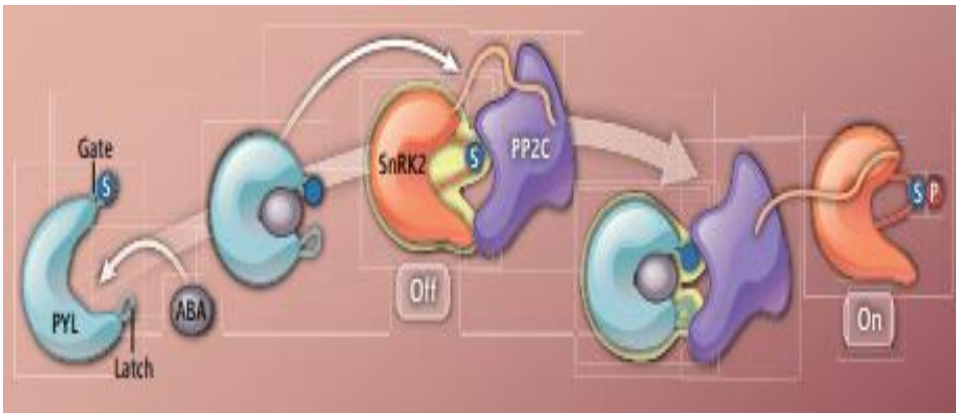

Figure 17 Molecular mimicry between the kinase SnRK2 and the hormone receptor PYL bound to ligand ABA permits alternate binding to the PP2C phosphatase. This change in partners activates (on) or deactivates (off) SnRK2, allowing it to phosphorylate downstream signals.
Source Leung, (2012).

On the other hand, when ABA binds to the ABA receptors family PYR/PYL/RCAR, this allows the bounds of the receptors in the catalytic site of PP2Cs to inhibit their enzymatic activity. In that case, ABA-induced inhibition of PP2Cs that leads to SnRK2 activation (Boudsocq et al., 2007; Soon et al., 2012; Leung, 2012).

A crucial event in the receptor's activation was found to be an open-to-closed conformational change in the gate loop of the receptor protein. More recent progress has provided strategies for controlling the gate's closure using chemical agonists (Melcher et al., 2010; Todoroki; Hirai, 2002) or protein engineering approaches. On the other hand, ABA antagonist could be used inhibiting ABA signaling in vivo and further investigations using this approach may reveal the function of ABA in diverse plant species. ABA antagonists may provide new insights into the function of ABA in desiccation tolerance during the evolution of plants on land (Takeuchi et al., 2014).

1.7 Evolution of ABA sensing and signaling

ABA is ubiquitious in plants and it is also produced by some phytopathogenic fungi, bacteria and metazoans ranging from sea esponges to humans (Wasilewska et al., 2008). Based on the available fossil record, the first land plants (embryophytes) colonized the terrestrial habitat about 500 million to 470 million years ago (Sanderson et al., 2004; Lang et al., 2010). Regarding cellular dehydration in plants, the core ABA signaling components found in *Arabidopsis* are conserved only in land plants (Figure 18), unlike the auxin and ethylene signaling components (Klingler et al., 2010; Umezawa et al., 2010; Hauser et al., 2011), supporting the idea that ABA signaling components may have played a crucial role in land colonization by plants. Furthermore, phylogenetic and transcriptome data suggest that plants have developed a highly sophisticated stress tolerance system through the expansion of duplicate gene families implicated in ABA signaling (Hanada et al., 2011).

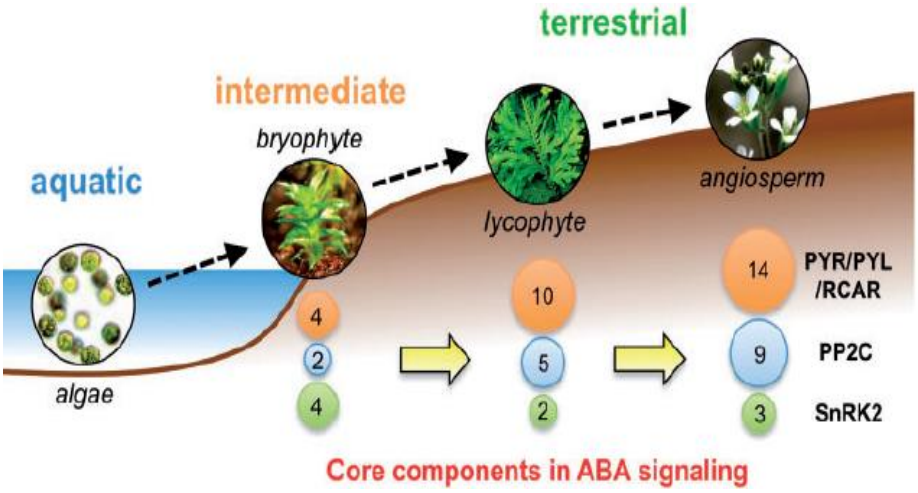

Figure 18 Evolution of core components of ABA signaling. The PYR/PYL/RCAR, group A PP2C and subclass III SnRK2 are conserved from bryophytes. The development of an ABA signaling system seems to be highly correlated with the evolution from aquatic to terrestrial plants. As representatives, component numbers of bryophyte, lycophyte and angiosperm were obtained from *Physcomitrella patens*, *Selaginella moellendorffii* and *Arabidopsis thaliana*, respectively.

Source Umezawa et al., (2010).

ABA was characterized like an important endogenous small molecule that mediates stress-responsive gene expression, stomatal closure, and vegetative growth modulation (Rodriguez-Gacio et al., 2009) in water deficit conditions. Overall, the core ABA signaling components play an essential role in both fast and slow response to cellular dehydration (Figure 19). To maintain water, ABA promotes stomatal closure through the control of membrane transport systems (Osakabe et al., 2014), shoot growth is inhibited whereas the root growth rate is maintained to gain access to water (Des Marais et al., 2012). Thus, fast ABA signaling involves stomatal closure responses in guard cells, whereas the comparatively slow signaling pathways involve transcriptional regulation in both seeds and vegetative tissues (Miyakawa et al., 2013).

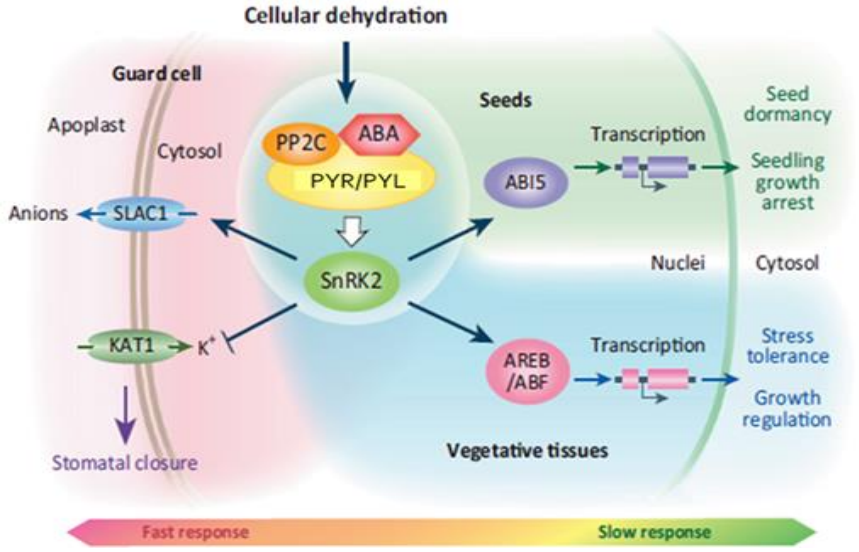

Figure 19 Current model for the major abscisic acid (ABA) signaling pathways in response to cellular dehydration. Core ABA signaling components [ABA, ABA receptors, protein phosphatases 2C (PP2Cs), and subclass III sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2s)] control both fast and slow ABA signaling pathways in response to cellular dehydration. Fast signaling involves stomatal closure responses in guard cells, whereas the comparatively slow signaling pathways involve transcriptional regulation in both seeds and vegetative tissues.

Source Adapted from Miyakawa et al., (2013).

In guard cells, SnRK2 protein kinases activate the anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) and inhibit the cation channel POTASSIUM CHANNEL IN ARABIDOPSISTHALIANA 1 (KAT1) through phosphorylation to release anions, causing stomatal closure (Cutler et al., 2010). In seeds, the post-germination phase induce cellular dehydration (Fujita et al., 2012) which cause an increase in plant ABA content through increase ABA synthesis in vascular tissues, adjustment of ABA metabolism (Nambara; Marion-Poll, 2005), and transport to sites of ABA action (Kanno et al., 2012). In roots, ABA signaling plays an important role to regulate root growth and root system architecture and this system is required for both hydrotropism and osmoregulation of water-stressed roots (SHARP et al., 2004; Gonzalez-Guzman et al., 2014). So, to regulate ABRE-dependent gene expression in seeds and vegetative tissues, respectively subclass III SnRK2s released from inhibition by PP2Cs activate ABA-INSENSITIVE 5 (ABI5) and ABA-responsive element (ABRE) binding protein (AREB)/ABRE-binding factor (ABF) transcription factors (TFs) (Miyakawa et al., 2013).

1.7.1 The tripartite system: PYL-PP2C-SnRK complex

Abscisic acid (ABA) has a central role regulating adaptive responses in plants (Gonzalez-Guzman et al., 2014). Under drought, this phytohormone, synthesized in roots and leaves during periods of water scarcity (Thompson et al., 2007), is perceived by ABA receptors that are the first component of the ABA tripartite systems (Klingler et al., 2010). Further, the PYL-ABA complex bind to the clade A phosphatase type 2C (PP2C) inactivating them (Hao et al., 2011; Ma et al., 2009; Park et al., 2009). Then, the subclass III SNF1-related kinase (SnRK2) proteins are activated by dephosphorylation allowing expression of downstream stress responsive genes (Cutler et al., 2010). In this system, SnRK2 and PP2C

proteins function therefore as positive and negative regulators of ABA pathway, respectively.

1.7.2 PYR-PYL/RCARs: ABA receptors:

Concerning ABA receptors, PYR/PYL/RCAR proteins are members of the large superfamily of soluble ligand-binding proteins defined as the START-domain superfamily (Iyer et al., 2001), more recently named Bet v I-fold superfamily (Radauer et al., 2008). After the genetic and biochemical identification of PYL/RCARs, several groups have determined the protein structure of the complex between PYL/RCARs and PP2Cs via X-ray crystallography. To date, the crystal structures of PYR1 (Nishimura et al., 2010; Santiago, et al., 2009a), PYL1 (Miyazono et al., 2009), PYL2 (Melcher et al., 2009; Yin et al., 2009), PYL3 (Zhang et al., 2013; Zhang et al., 2012), PYL5 (Zhang et al., 2013), PYL9 (Zhang et al., 2013; Nakagawa et al., 2014), PYL10 (Hao et al., 2011; Sun et al., 2012), and PYL13 (Li et al., 2013) have been reported.

Cellular ABA receptor PYL/RCAR orthologs appear to be highly evolutionarily conserved in plants. For example, the *A. thaliana* genome encodes 14 PYR/RCAR proteins, named PYR1 and PYR1-like (PYL) 1-13 or RCAR1-RCAR14 (Ma et al., 2009; Park et al., 2009). The receptor family can be classified into different sub-types based on the sequence similarity, ABA sensitivity, oligomeric state, basal activation level and function. For instance, PYR1/RCAR11, PYL1/RCAR12, PYL2/RCAR14 and PYL3/RCAR13 proteins of *Arabidopsis*, which form homodimers in the absence of ABA, were released as monomers following ABA binding and subsequently interacted with group-A PP2Cs. In contrast, PYL4/RCAR10, PYL5/RCAR8, PYL6/RCAR9, PYL8/RCAR3, PYL9/RCAR1 and PYL10/RCAR4 behave as monomers in both the presence and absence of ABA, and these monomers can inhibit group-A

PP2Cs regardless of ABA binding (Yoshida et al., 2015). There are at least 10 functional orthologs in *Oryza sativa* (Kim et al., 2012), 14 in *Solanum lycopersicum* (Sun et al., 2011; Gonzalez-Guzman et al., 2014), 7 in *Vitis vinifera* (Boneh et al., 2012) and 6 in *Citrus sinensis* (Romero et al., 2012).

A series of mutations in *PYR1/RCAR11* increase its basal activity. Once the combination of these mutations was incorporated into *PYL2* this was sufficient for the activation of ABA signaling in seeds (Mosquna et al., 2011) suggesting that a single receptor modified is sufficient to activate this signaling. In this sense, a useful tool that activate individual family members selectively and explore phenotypic consequences (Ben-Ari, 2012).

ABA receptors *PYL4* and *PYL5* are known to be involved in the regulation of *ABI1* and *ABI2* genes, ABA normally lowers wild type PP2C activity via PYR/PYL proteins, but ABI PP2Cs escape this and disrupt signaling due to their residual activity (Park et al., 2009). Furthermore, *PYL4* and *PYL5* have been pointed as components of the crosstalk between the JA and ABA signaling pathways (Figure 20) (Lackman et al., 2011). In *N. tabacum* and *A. thaliana*, the *PYL4* gene is regulated by JA. The loss-of-function mutants in *PYL4* and *PYL5*, which were hypersensitive to JA treatment, showed reduced growth in comparison to wild type plants of *A. thaliana*. Both mutants *pyl4* and *pyl5* displayed reduced anthocyanin accumulation in response to JA compared to wild type (Lackman et al., 2011). Interestingly, *PYL4* and *PYL5* stand out among the genes that were up-regulated at 3 hours after under drought and inoculation by *Pieris rapae* (Davila Olivas et al., 2016) showing that these genes could act in different hormonal pathways intermediating both abiotic and biotic stresses. The *OsPYL/RCAR5* gene stands out as positive regulator of the ABA signal transduction pathway in seed germination and early seedling growth (Kim et al., 2012).

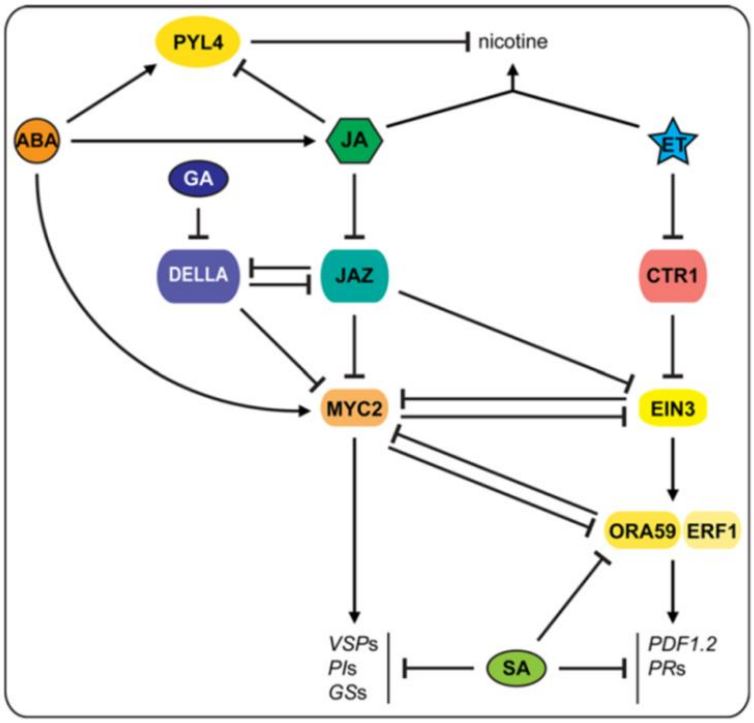

Figure 20 Schematic representation of interactions between hormonal cascades regulating induced defense against biotic agents. Insect herbivores induce JA-dependent MYC2 regulation of defense-related genes, which is enhanced by ABA signaling. Necrotrophic pathogens induce JA/ET-dependent signaling to regulate ERF1 and ORA59 and downstream defense-related genes. The two branches of defense responses mutually antagonize one another. GA and SA signaling generally inhibit JA-dependent defense responses.
Source Nguyen et al., (2016).

In *Arabidopsis* the overexpression of *PYL9/RCAR1*, *PYL5/RCAR8* and PYL8/RCAR3 genes produced enhanced ABA responses or elevated drought tolerance Several recent studies have suggested that the role of PYL8/RCAR3 is overlapping with but distinct from that of other PYR/PYL/RCAR. The PYL8/RCAR3 interacts with transcription factors such as MYB77 which lead to the transcriptional activity of MYB77 which modulates auxin signaling during lateral root development (Shin et al., 2007).

The RCAR7/PYL13 family member regulated the phosphatase activity of the PP2C ABI1, ABI2, and PP2CA proteins *in vitro* at nanomolar ABA levels. However, it appeared to differ from the majority of other RCARs once it failed to bind to the hypersensitive to ABA 1 (HAB1) PP2C in a heterologous system (Bhaskara et al., 2012). Of the 14 RCARs, it has been shown that RCAR7 was the only one that had a variant ABA-binding pocket, with three non-consensus amino acids (Fuchs et al., 2013).

Despite ABA receptor function of RCAR7 has been questioned it was recently demonstrated and the structural constraints that contribute to specific pairing of RCAR7 with PP2Cs was identified (Fuchs et al., 2013).

1.7.3 PP2Cs phosphatases

Otherwise, protein phosphatases are already well known to function as negative regulators of ABA signaling pathway. The physiological functions of PP2Cs were clearly determined genetically in the beginning of XXI century (Umezawa et al., 2010a). Model plants such as *A. thaliana* and rice contained for example 80 and 78 PP2C genes, respectively (Xue et al., 2008). Phylogenetic analyses from *Arabidopsis* and soybean were supported by gene structure and protein motifs and led to subdivide the *PP2C* genes (Figure 21).

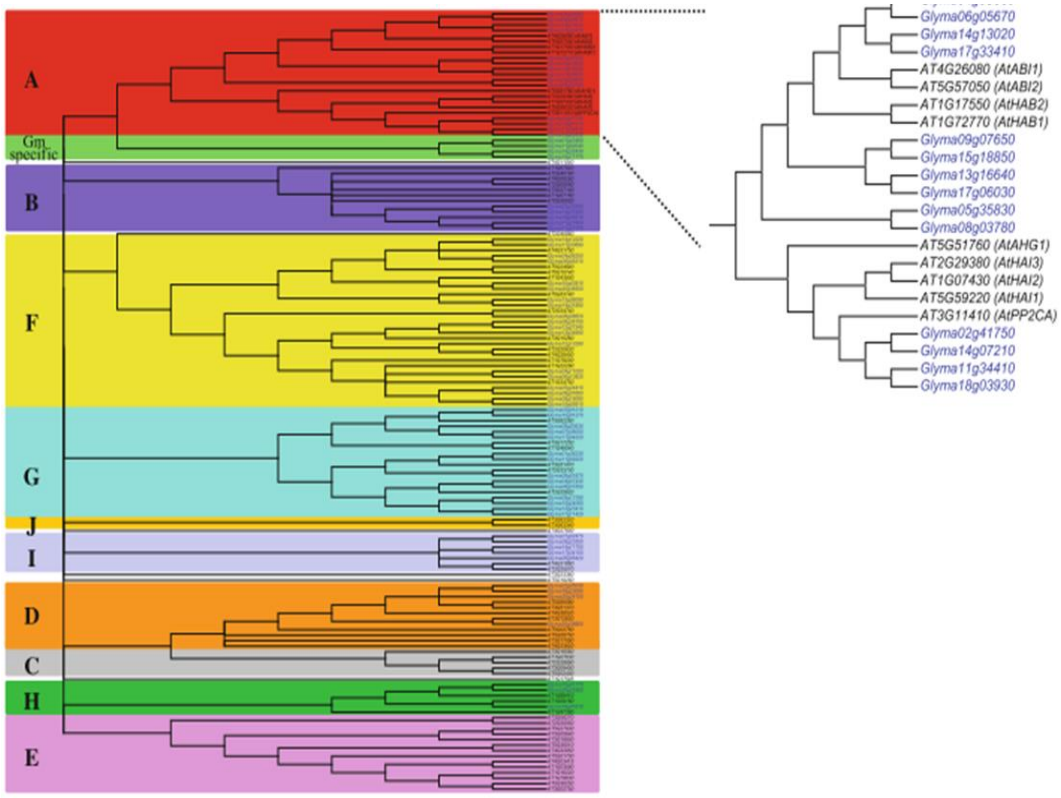

Figure 21 An unrooted phylogenetic tree based on sequence alignment of the catalytic domains encoded by soybean and *Arabidopsis* PP2C. Each cluster was categorized according to the phylogenetic analysis of *Arabidopsis* PP2C genes (SCHWEIGHOFER et al., 2004). The cluster of *Arabidopsis* (black font) and soybean (blue font) group A PP2C is enlarged.
Source Adapted from Ben-Ari et al., (2012).

PP2C proteins are classified according to the substrate into Ser/Thr, Tyr or dual-specificity classes. Depending on their biochemical and structural features, plant Ser/Thr phosphatases are further divided into PP1, PP2A and PP2C groups (Luan, 2003). The PP2C proteins contain both catalytic and regulatory domains (Figure 22) within the same polypeptide chain (Shi, 2009).

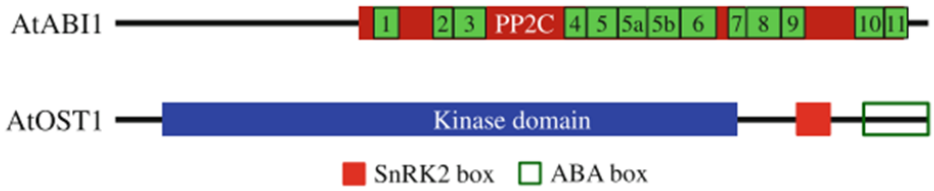

Figure 22 A schematic representation of the group A PP2C, AtABI1 and the SnRK2, AtOST1. AtABI1consit of a PP2C (catalytic) domain (brown) in addition to the 11 motifs (green) (BORK et al., 1996) at its C-terminal. AtOST1 consist of a kinase domain (blue) at its N-terminal followed by a SnRK2 box (red) and an ABA box (green). The ABA box appears with an empty green box to emphasize that this domain is not used for SnRK2 identification.
Source Adapted from Ben-Ari (2012).

Gene duplication analyses reveals that whole genome and chromosomal segment duplications mainly contributed to the expansion of both *OsPP2C*and *AtPP2C* genes, however, tandem or local duplication occurred less frequently in *Arabidopsis* than rice (Xue et al., 2008).

PP2C phosphatases belong to the Mn^{2+}/Mg^{2+} metal-dependent protein phosphatases PPM family and negative regulatory roles of PP2C subgroup A in ABA signaling have been demonstrate after 2009 and suggesting that PP2C functions are well conserved in different plant species (Saez et al., 2003; Komatsu et al., 2009). Two homologous members of clade B PP2Cs were also reported to be involved in ABA signaling (Ben-Ari, 2012). Regarding, group-A PP2Cs are functionally redundant at the molecular level, but they have distinctive roles in different tissues and organs, as indicated by tissue-specific expression patterns (Umezawa et al., 2010). The PP2C functions emphasized the existence of sophisticated signaling pathways in plants, in which protein dephosphorylation played a crucial role towards determining specificities (Schweighofer et al., 2004).

At least six *A. thaliana* PP2Cs belonging to the group A act as negative regulators of the ABA pathway (Gosti et al., 1999; Merlot et al., 2001; Leonhardt

et al., 2004; Saez et al., 2003; Yoshida et al., 2006). On the other hand, ten *VvPP2Cs* and two *CsPP2C* were identified in *V. vinifera* and *C. sinensis* from group A, respectively, while a family of 23 group A-PP2C genes was found in *S. lycopersicum* consisted in a family of (Wang et al., 2013a). In *V. vinifera* and *C. sinensis* all these genes were shown to be up-regulate in response to drought (Gambetta et al., 2010; Boneh et al., 2012a). Interestingly, the expression pattern of the OsPP2C subfamily A genes plants treated with ABA, salt, osmotic (mannitol) and cold stress is in good agreement with the microarray data for *Arabidopsis* subfamily A members, suggesting that the members of this subfamily play foremost roles in ABA-mediated processes related to stress responses both in monocots and eudicots (Xue et al., 2008).

1.8 SnRK2 kinases

The reversible phosphorylation of proteins is a fundamental mechanism by which living organisms modulate signal transduction events (Cutler et al., 2010). Once active, SnRK2 kinases can phosphorylate downstream effectors (Figure 23) such as the basic leucine zipper transcription factors ABFs/AREBs, thus switching-on the transcription of ABA-responsive genes (Furihata et al., 2006).

The first positive regulators termed SnRK2 (Subfamily 2 of sucrose non-fermenting 1 related protein kinases SNF1) gene was isolated and characterized 20 years ago in wheat and called PKABA1 (Anderberg; Walker-Simmons, 1992). At least 10 SnRK2-encoding genes were found in *A. thaliana* genome, with *SnRK2.2, SnRK2.3* and *SnRK2.6* being associated with ABA signaling (Fujii; Zhu, 2009). The entire *SnRK2* gene family was also identified in many crops such *O. sativa* (Kobayashi et al., 2004), *S. lycopersicum* (Sun et al., 2011; Sato et al., 2012; Wang et al., 2013), *V. vinifera* (Boneh et al., 2012) and *C. sinensis* (Romero et al., 2012).

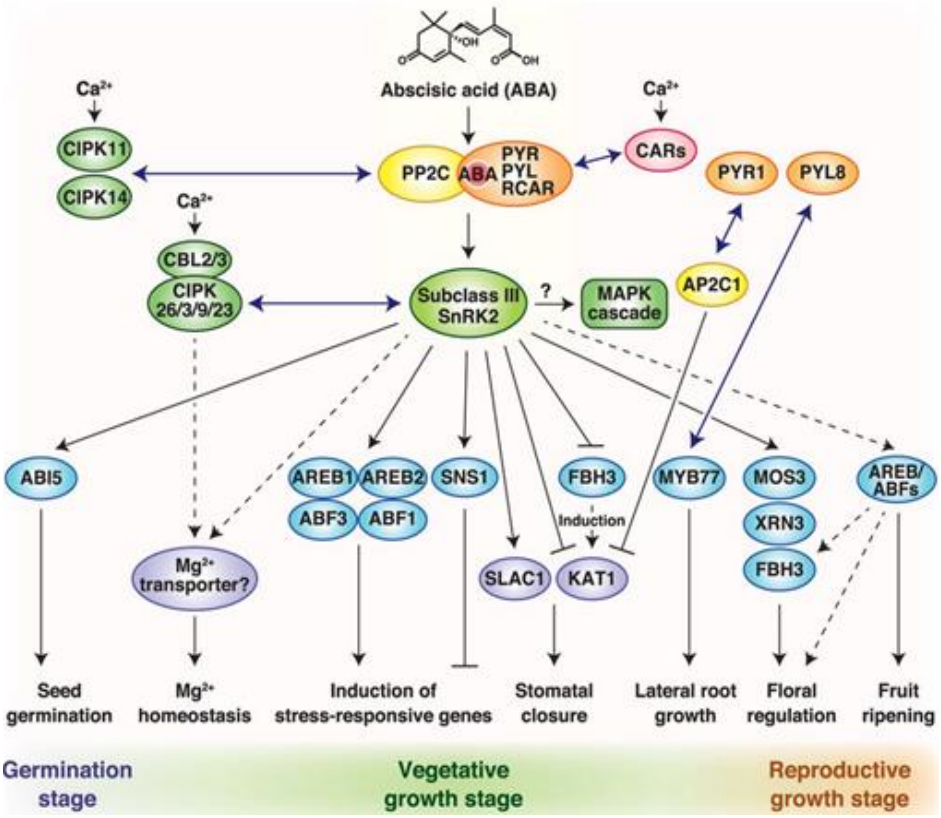

Figure 23 Schematic model of the ABA signaling pathway, which is mediated by novel signaling components discovered in recent omics studies as well as by the core components PYR/PYL/RCAR, group-A PP2Cs and subclass III SnRK2. In addition to the core components, several protein kinases/protein phosphatases (green and yellow ellipses, respectively) are key players in the regulation of ABA-mediated physiological responses during the life cycle of plants. Several PYR/PYL/RCAR proteins (represented by orange ellipses) are also able to regulate ABA responses independent of group A PP2Cs. C2-domain ABA–related (CAR) proteins are shown as pink ellipses. Downstream targets involved in transcriptional regulation and ion transport are shown as blue and purple ellipses, respectively. Physical interactions identified by interactome analyses are depicted as bidirectional blue arrows. The dashed lines indicate possible but unconfirmed routes. Due to space constraints, not all interacting protein and /or substrates of the core components are shown.

Source Adapted from Yoshida et al., (2015).

Among SnRK superfamily proteins, SnRK2s plays a major part in ABA signaling and it were divided into three subclasses (Figure 24), which differed by their activation in response to ABA (Kobayashi et al., 2004; Boudsocq et al., 2004). Subclass I corresponded to genes not activated in the presence of ABA. On the other hand, SnRK2s proteins of subclass II were activated to a lesser extent by ABA. In turn, those of subclass III are strongly activated by ABA.

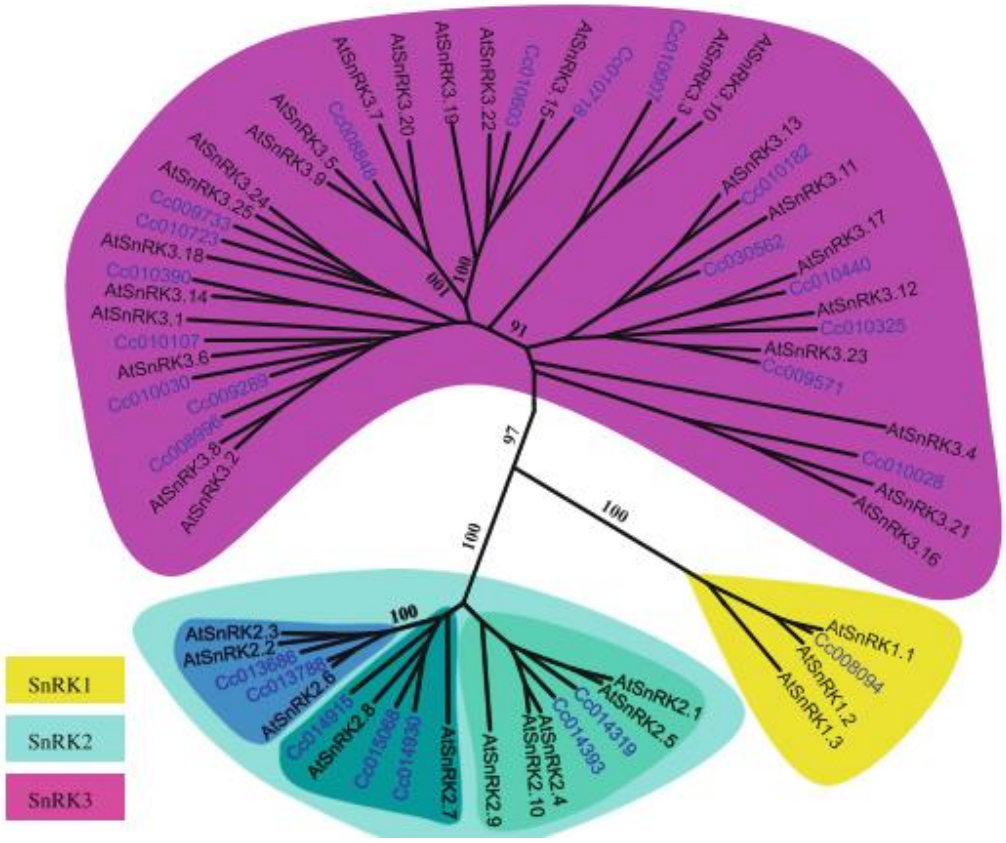

Figure 24 All SnRKs from *Arabidopsis* (black font) and Clementine (blue font) are presented with yellow (SnRK1), blue (SnRK2) and purple (SnRK3) backgrounds. The SnRK2s were clustered into three subgroups, each of which appears with a different background color.

Source Ben-Ari, (2012).

The C-terminal extremity of SnRK2 subclass III contain an Asp-enriched domain required for both the hormone specific activation of the kinase (Belin et al., 2006) and interaction with PP2C (Hubbard et al., 2010). Domain I represent the SnRK2 box, which is conserved in all members of the SnRK2 gene family. The kinase domain presents an ATP-binding and the activation loop. Domain II is ABA box is conserved only in subclass III of the SnRK2 gene family.

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PRESENTATION OF THE PHD PROJECT

Regarding the key roles of PYL/SnRK2/PP2C tripartite system in higher plants, the following scientific questions arisen concerning coffee:

- how many genes composed the PYL/SnRK2/PP2C tripartite system in coffee and how they are organized?
- are these genes expressed in the same manner in different coffee tissues and organs?
- are they differentially expressed in D^T and D^S clones and cultivars of coffee under drought and ABA?
- Does it exist different expression profiles of these genes in *C. arabica* and *C. canephora*?
- Is it possible to identify alleles for improving drought tolerance in *C. canephora* for use in breeding programs?
- is it possible to correlate the diversity of these genes with coffee evolution and adaptation?

In order to get the answers to these questions, the main objectives of this work were:

(i) to identify the candidates genes coding for the ABA proteins receptors (PYR/PYL/RCAR), the phosphatases (PP2C) and kinases (SnRK2) proteins involved in the first steps of ABA signalling pathways in *C. canephora*;

(ii) to characterize these *C. canephora* genes, comparing their families and structure with those described in model plants;

(iii) to identify the functional *C. canephora* PYL/PP2C/SnRK2 orthologs;

(iv) to characterize the expression profile of genes belonging to the tripartite system (PYR/PYL/RCAR-PP2C-SnRK2) in leaves and roots of D^T and D^S clones of *C. canephora* submitted or not to drought stress;

(v) to compare these expression profiles to those obtain in *silico* in different *C. canephora* tissues;

(vi) to study the effects of exogenous ABA on the gene expression of these genes;

(vii) The results obtained regarding these questions are presented in the following chapters.

CHAPTER 1

The PYL/PP2C/SnRK2 tripartite system in *C. canephora*

THE PYL/PP2C/SNRK2 TRIPARTITE SYSTEM IN *C. CANEPHORA*

The increased availability of plant genome data is essential to perform comparative and functional genomic research with insights in plant evolution which can greatly expand the knowledge of the molecular basis of abiotic stress responses in *C. canephora.*

Comparative genomics studies has shown that ABA regulation in water-stress responses is functionally conserved throughout the land plant lineage, from bryophytes, that diverged from the land plant lineage some 450 million year ago (MYA), to angiosperms (usually represented by *A. thaliana*) that first appeared between 150 and 250 MYA (Doyle, 2012). An essential conservation of responses between the earliest lineages and the flowering plants is clear from studies of the consequences of ABA treatment, or the application of osmotic and drought-stress.

According to Ben-Ari (2012), the identification of orthologs using *A. thaliana* as reference is an excellent approach for functional studies and comparative genomics once *Arabidopsis* is the best studied model species for high plants. Besides phylogenetic considerations, Rubiaceae and Solanaceae are frequently considered as "sisters" plant families based on genetic similarities observed between *C. canephora* and *S. lycopersicum* (Guyot et al., 2012), such as genome size (Noirot et al., 2003; Van der Hoeven, 2002), the basic chromosome number, the cytogenetic chromosome architecture (Pinto-Maglio; Da Cruz, 1998; Hamon et al., 2009), the absence of polyploidization (Wu et al., 2010) and expressed genes in the seed and cherry (Lin et al., 2005). The structural relationships between *C. canephora*, *S. lycopersicum* and *V. vinifera* genomes were carried-out by Guyot et al. (2012) aiming to evaluate the genome conservation and evolution combining comparative mapping at the macro and micro-scale levels. These studies showed that *Solanaceae* microstructures appear much more different than the conservation between *C. canephora* and *V. vinifera*

tree, suggesting a divergent and specific evolution of the locus in the *Solanaceae* prior to the separation with the *Rubiaceae*.

Recently, a high-quality draft genome of *C. canephora* was generated which displays a conserved chromosomal gene order among asterid angiosperms (Denoeud et al., 2014). Although there is no sign of the whole-genome triplication as identified in *Solanaceae* species such tomato, the genome includes several species-specific gene family expansions.

In the last years, great efforts have been implemented in genomics to attempt to understand the genetic determinism of tolerance to environmental stresses, biotic and abiotic, especially in model species (Umezawa et al., 2006; Ashraf, 2010). The same applies to coffee for which the recent progress in DNA sequencing methods, genetics and biotechnology permitted the identification of thousands EST sequences (Lin et al., 2005; Poncet et al., 2006; Vieira et al., 2006; Vidal et al., 2010; Mondego et al., 2011), the recent complete genome sequence of *C. canephora* (Denoeud et al., 2014). the construction of genetic maps (Lefebvre-Pautigny et al., 2010, Leroy et al., 2011) and the improvement of genetic transformation techniques (Ribas et al., 2011) These scientific advances now paved the way to investigate the structure of complex gene families in this plant, as it is the case for the genes coding for the proteins of the PYL/PP2C/SnRK2 tripartite system.

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Article

MOLECULAR MECHANISMS OF ABA-MEDIATED RESPONSE TO DROUGHT IN LEAVES AND ROOTS OF *COFFEA CANEPHORA*.

Running title: ABA-mediated response to drought in *Coffea canephora*

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ABSTRACT

Abscisic Acid (ABA) pathway is an ancient signaling universally conserved in land plants which coordinates several aspects of the plant response to water deficit such as root architecture, seed dormancy and stomatal regulation. A mechanism of ABA signal transduction has been proposed, evolving intracellular ABA receptors (PYR/PYL/RCARs) interacting with PP2Cs phosphatases and SnRK2 protein kinases. The goal of this study was to identify and characterize for the first time the orthologs of this tripartite system in *C. canephora*. For this purpose, protein sequences from Arabidopsis, citrus, rice, grape, tomato and potato were chosen as query to search orthologous genes in the Coffee Genome Hub (http://coffee-genome.org/). Differential expression in leaves, seeds, roots and floral organs was checked through *in silico* analyses. *In vivo* gene expression analyses were also performed by RT-qPCR in leaves and roots of drought-tolerant (D^T 14, 73 and 120) and -susceptible (D^S 22) *C. canephora* Conilon clones submitted to drought. This approach allowed the identification and characterization of 17 candidate genes (9 PYL/RCARs, 6 PP2Cs and 2 SnRK2s) in *C. canephora* genome. The protein motifs identified in predicted coffee sequences enabled to characterize these genes as family's members of receptors (PYL/RCARs), phosphatases (PP2Cs) or kinases (SnRK2s) of the ABA response pathway. These families were functionally annotated in the *C. canephora* genome. *In vivo* analyses revealed that eight genes are up-regulated under drought

conditions in both leaves and roots tissues. Among them, three genes coding phosphatases were expressed in all clones therefore suggesting that they were activated as a general response to cope with drought stress. However, two other phosphatase coding genes were up-regulated only in the D^T clones, suggesting that they may constitute key-genes for drought tolerance in these clones. The D^T clones also showed differential gene expression profiles for five other genes therefore reinforcing the idea that multiple biological mechanisms are involved drought tolerance in *C. canephora*.

INTRODUCTION

The first bitter mouthful in the morning which gives daily energy to the planet is coffee, the major tropical commodity traded worldwide and source of income for many developing countries (Lashermes et al., 2008). With about a third of the world production, Brazil is the first coffee producing country (ICO, 2016). Coffee production is subject to regular fluctuations mainly due to adverse climatic conditions, such as prolonged drought periods. Based on the last report of the Intergovernmental Panel on Climate Change (IPCC), the increase of temperature and drought periods would change the distribution of coffee production zones worldwide leading to environmental, economic and social problems (Davis et al., 2012; Bunn et al., 2015; Ovalle-Rivera et al., 2015) as well as an increase in pests and diseases (Jaramillo et al., 2009; Magrach & Ghazoul, 2015). Drought is a key

factor affecting coffee plant development and production (DaMatta and Ramalho, 2006), bean biochemical composition (Vinecky et al., 2016) and quality (Silva et al., 2005).

Among the known 124 perennial species in the coffee genus (Davis et al., 2011), the commercial coffee production concerns only two species, *Coffea canephora* and *C. arabica*. While *C. canephora* is allogamous and diploid (2n=2x=22), *C. arabica* is an autogamous allotetraploid species (2n=4x=44) coming from a natural hybridization between *C. canephora* and *C. eugenioides* ancestrors (Lashermes et al., 1999). Concerning drought tolerance, it is well known that genetic variability exists within *C. canephora* species, the Guinean and SG1 sub-group of Congolese being more tolerant to drought than Congolese plants of SG2 sub-group (Montagnon & Leroy, 1993). Such diversity also exists in Conilon plants of *C. canephora* cultivated in Brazil that are closely related to the SG1 group (Montagnon et al., 2012). Among the strategies commonly observed in coffee plants to cope with water limitation are leaf folding and inclination that reduce water loss and exposure to high irradiance. During the last decade, several drought-tolerant (D^T) and susceptible (D^S) clones of Conilon were identified and previously characterized physiologically (Lima et al., 2002; DaMatta et al., 2003; Pinheiro et al., 2004; Praxedes et al., 2005). At the molecular level, genes differentially expressed under drought were also identified in leaves of D^T and D^S clones of *C. canephora* (Marraccini et al., 2011, 2012; Vieira et al.,

2013), some of them (e.g. *RD29* and *DREB1D*) being linked to ABA-dependent pathways.

It is well known that abscisic acid (ABA) has a central role regulating the adaptive response to drought tolerance in plants (Gonzalez-Guzman et al., 2014). Under stress conditions, this phytohormone, synthesized in roots and leaves during periods of water depletion (Thompson et al., 2007), is perceived by PYR/PYL/RCAR receptors that are the first component of the ABA tripartite systems (Klingler et al., 2010). Once formed, the PYL-ABA complex bind to the clade A phosphatase type 2C (PP2C) inactivating them (Hao et al., 2011; Ma et al., 2009; Park et al., 2009). Then, the subclass III SNF1-related kinase (SnRK2) proteins are activated by dephosphorylation allowing expression of downstream stress responsive genes (Cutler et al., 2010). In this system, SnRK2 and PP2C proteins function therefore as positive and negative regulators of the ABA pathway, respectively.

Using the recently published genome sequence of *C. canephora* (Denoeud et al., 2014), the main objective of this work was (i) to identify the orthologous genes belonging to the tripartite system (*PYL-PP2C-SnRK2*) of ABA in *C. canephora,* , (ii) to characterize these orthologs according to gene structure, protein functional domains, phylogeny, synteny and (iii) to evaluate the expression profile of those genes in leaves and roots of contrasting (D^T and D^S) clones *C. canephora* submitted or not to drought conditions.

MATERIAL AND METHODS

Plant material

Drought-tolerant (D^T: 14, 73 and 120) and -susceptible (D^S: 22) clones of *C. canephora* Conilon were grown in greenhouse conditions (under controlled temperature 25°C, relative humidity of 70% and photosynthetic flux PPF 900 $\mu mol^{-2}s^{-1}$) at UFV (University of Viçosa-UFV, Minas Gerais, Brazil). At 6 months old, drought stress was applied to the plants by water withdrawal (NI: non-irrigated) to reach a predawn leaf water potential (Ψ_{pd}) of around -3.0 MPa. From each clone, biological triplicate samples (leaves and roots) were collected in both irrigated (I: control) and NI conditions, immediately frozen in liquid nitrogen and stored at -80°C for RNA extractions and ABA quantification.

Genomic data

Genomic data from a double haploid accession of *C. canephora* available in Coffee Genome Database (http://coffee-genome.org/, Dereeper et al. [2015]) were used as reference sequences.

In silico identification and characterisation of candidate genes of the PYR/PYL/RCAR-PP2C-SnRK2 tripartite system

PYR/PYL/RCAR-PP2C-SnRK2 orthologs genes from *C. canephora* , and their orthologs from Arabidopsis thaliana, Solanum lycopersicum, Solanum tuberosum, Vitis vinifera, Citrus sinensis and Oryza sativa were identified in the following databases: NCBI (http://www.ncbi.nlm.nih.gov/), TAIR (http://www.arabidopsis.org/), AtGDB (http://www.plantgdb.org/AtGDB/), Phytozome (http://www.phytozome.net/), Sol Genomics Network (http://solgenomics.net/), SlGDB (http://www.plantgdb.org/SlGDB/), GreenPhyl (http://www.phytozome.net/), Grape Genome Database (http://www.genoscope.cns.fr/externe/), Gramene Database (http://www.gramene.org/), Plant Genome Database (http://www.plantgdb.org/), Citrus Genome Database (http://www.citrusgenomedb.org/) and Rice Genome Annotation (http://rice.plantbiology.msu.edu). BLAST searches were carried out using these sequences as query against the Coffee Genome Database and Rubiaceae ESTs database (e-value < e^{-10}) to isolate coffee genes that were further translated to compare their corresponding proteins with proteins of other species using the MAFFT program (Katoh & Toh, 2008) available at South Green Platform (http://www.southgreen.fr/). The conserved amino acids were identified using the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/). Genes that did not contain specific domains were removed. Phylogenetic analyses were performed with orthologous sequences that were filtered with Gblocks (Castresana, 2000) and used to construct the phylogenetic trees using PhyML

algorithm (Guindon et al., 2010). To compare gene and species, reconciled trees were constructed using the RAP-Green algorithm (Dufayard et al., 2005) and the reference tree provided by the NCBI taxonomic database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy). The HMM (Hiden Markov Model) was used to build and validate the phylogenetic analyses that were visualized using the Dendroscope software (Huson et al., 2007). All candidate genes were functionally annotated in the Coffee Genome Database using Artemis software (Carver et al., 2012). Gene structures were predicted using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). The transcriptomic data available in the Coffee Genome Database were used to perform in silico expression analyses that were normalized using RPKM (Fig. S1). The gene duplication patterns were generated using the MCScanX software (Wang et al., 2012) and were formatted by Circos (http://circos.ca/) for graphical representation.

RNA extraction and real-time qPCR assays

Total RNAs were extracted from leaves and roots of *C. canephora* as previously described (Marraccini et al., 2011). Contaminant genomic DNA was eliminated from purified RNAs by RQ1 RNase-free DNase (Promega) treatment according to the fabricant. RNA integrity was verified by agarose gel electrophoresis with ethidium bromide staining. Synthesis of the first-strand

cDNA was done by treating 2.4µg of total RNA with the ImProm-II Reverse Transcription System and oligo (dT15) according to the manufacturer's recommendations (Promega). Real-time qPCR assays were carried out with the synthesized single-stranded cDNA using the protocol recommended for 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). cDNA preparations were diluted (1/20) and tested by qPCR using primer pairs (Table S1) designed using the Primer Express software (Applied Biosystems) and preliminarily tested for their specificity and efficiency against a cDNA mix from roots and leaves. The qPCR was performed with 1µl of diluted single-stranded cDNA and 0.2 µM (final concentration) of each primer in a final volume of 10µl with 1x SYBR green fluorochrome (SYBRGreenqPCR Mix-UDG/ROX, Invitrogen). The reaction was incubated for 2 min at 50°C and 5 min at 95°C (UDG step), followed by 40 amplification cycles of 3 s at 95°C, 30 s at 60°C. Data were analysed using the SDS 2.1 software (Applied Biosystems) to determine the cycle threshold (Ct) values. Specificity of the PCR products generated for each set of primers was verified by analysing the Tm (dissociation) of amplified products. Gene expression levels were normalized to expression level of ubiquitin (CcUBQ10) as a constitutive reference (Barsalobres-Cavallari et al., 2009). Expression was expressed as relative quantification by applying the formula $(1+E)^{-\Delta\Delta Ct}$, where $\Delta Ct_{target} = Ct_{target\ gene} - Ct_{reference\ gene}$ and $\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{internal\ calibrator}$.

ABA extraction and quantification

ABA was extracted from leaves and roots tissues of *C. canephora* clones stored at -80°C as previously mentioned (see plant material section). Initially, samples were lyophilised and ground to a power in liquid nitrogen. ABA was extracted (Berry & Bewley, 1992) and quantified by ELISA using the Phytodetek ABA test kit (Agdia, Elkhart, IN, USA).

Statistical analyses

The statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Identification of CcPYLs-PP2Cs-SnRK2s orthologs in C. canephora

The protein sequences of ABA receptors, phosphatases and kinases from *A. thaliana*, *C. sinensis*, *V. vinifera*, *S. lycopersicum*, *S. tuberosum* and *O. sativa* were used as query to identify orthologous genes in *C. canephora* through BLASTP (Table S2-S4), leading to the identification of 17 putative coffee proteins according to the analysis of their functional domains. Nine proteins were homologous to the PYR/PYL/RCAR (Fig. 1a), six to clade-A PP2C (Fig. 1b) and

seven putative coffee SnRK2 kinases belonging to subclass I and II (Fig.1c). Two additional SnRK2s of subclass III were also identified (Fig. 1d). These genes were named according to the results of phylogenetic analyses and sequence homology, as follows: *CcPYR1* (*Cc08_g02750*), *CcPYL2* (*Cc08_g10450*), *CcPYL4* (*Cc02_g05990*), *CcPYL7a* (*Cc00_g17440*), *CcPYL7b* (*Cc00_g23730*), *CcPYL8a* (*Cc02_g01800*), *CcPYL8b* (*Cc08_g15960*), *CcPYL9* (*Cc02_g39180*), *CcPYL13* (*Cc02_g15060*), *CcABI1* (*Cc08_g11010*), *CcABI2* (*Cc06_g11740*), *CcAHG2* (*Cc08_g16010*), *CcAHG3* (*Cc02_g07430*), *CcHAB* (*Cc04_g01620*), *CcHAI* (*Cc01_g13400*), *CcSnRK2.1* (*Cc00_g19320*), *CcSnRK2.2* (*Cc07_g05710*), *CcSnRK2.6* (*Cc02_g18420*), *CcSnRK2.8* (*Cc10_g06790*), *CcSnRK2.8* (*Cc07_g14700*), *CcSnRK2.10* (*Cc02_g22790*), *CcSnRK2.11* (*Cc08_g11200*), *CcSnRK2.12* (*Cc00_g35430*) and *CcSnRK2.13* (*Cc00_g07830*).

Most of *CcPYLs-PP2Cs-SnRK2s* genes were found in chromosome 2 of *C. canephora* (Fig. 2a). Regarding *PYR/PYL/RCAR* gene family, the *CcPYL7a* and *CcPYL7b* genes were located on the chromosome 0. The seven others *CcPYLs* genes were on the chromosomes 2 (*CcPYL4*, *CcPYL8a*, *CcPYL9* and *CcPYL13*) and 8 (*CcPYR1*, *CcPYL2* and, *CcPYL8b*). The six clade-A *PP2Cs* genes were positioned on five different chromosomes: *CcHAI* in chr1, *CcAHG3* in chr2, *CcHAB* in chr4, *CcABI2* in chr6 and *CcABI1* and CcAHG2 in chr8. The *CcSnRK2.6* and *CcSnRK2.2* of subclass III were located on the chr2 and chr7, respectively. For the seven *SnRK2* genes of subclasses I and II, *CcSnRK2.1*,

CcSnRK2.12 and *CcSnRK2.13* were located on the chr0 whereas *CcSnRK2*.10, *CcSnRK2*.8, *CcSnRK2*.11 and *CcSnRK2*.8 were in chr2, chr7, chr8 and chr10, respectively.

Functional annotation of CcPYL-PP2C-SnRK2 genes

The 24 genes of the coffee tripartite system were functionally annotated on *C. canephora* genome (Fig. 2b). The occurrence of duplication events in the *CcPYL-PP2C-SnRK2* gene families was investigated through analyses of the paralogous regions. These analyses showed that CcPYL proteins shared high identity with ABA receptors from grape, while the CcPP2Cs were closely related to tomato and potato phosphatases, and CcSnRK2s with citrus kinases (Table S2-S4). Except the CcSnRK2.12 and CcSnRK2.13 proteins which not contained all domains (Fig. 1c), the lengths of CcPYL, CcPP2C and CcSnRK2 protein sequences were between 174-231, 418-546 and 336-363 amino acids, respectively (Fig. 1). The phylogenetic trees showed that PYL receptors and SnRK2 proteins were distributed in the three main subfamilies (Fig. 3a and 3b, Fig. S2 and S4). The putative protein sequences of *CcPYL7a*, *CcPYL7b*, *CcSnRK2.1*, *CcSnRK2.12-13* coding-genes located on chr0 were not showed on the resumed phylo-analyses, however, they are represented in the complete ones (Fig. S2 and S4).

ABA (PYR/PYL/RCAR) receptors

Among the nine PYR/PYL/RCAR proteins, CcPYR1, CcPYL8a and CcPYL9 showed high sequence identity (72%, 83% and 84%) with tomato sequences while CcPYL2 and CcPYL4 shared 84% and 74% of identity with the potato proteins, and CcPYL7a and CcPYL7b had 54% and 53% of identity with the same grape locus while the CcPYL8a and CcPYL8b proteins shared 82% of identity. Finally, the CcPYL13 showed 62% of sequence identity with grape GSVIVG01013161001 protein. BLASTP results showed that CcPYL4, CcPYL7a and CcPYL7b proteins were highly homologous to AtPYL6 from *A. thaliana*, CsPYL5 from *C. sinensis* and VvRCAR6 from *V. vinifera*, respectively (Table S2). The CcPYL4, CcPYL7a and CcPYL7b proteins also shared high identity respectively with the Solyc10g076410, Solyc10g085310 and Solyc03g095780 proteins of *S. lycopersicum*. All these coffee PYR/PYL/RCAR proteins (including in CcPYL7a and CcPYL7b located on chr0), contained key amino acid residues involved of both gate and latch loops conserved in ABA receptors (Fig. 1a).

The seven mapped CcPYL genes were identified on different ancestral blocks of the seven eudicot chromosomes such as the G2 (CcPYR1, CcPYL8a, CcPYL8b and CcPYL9), G4 (CcPYL2), G6 (CcPYL4) or G7 (CcPYL13) groups. All CcPYLs genes identified on the G2 ancestral block were located at the edges of their respective chromosomes. In addition of being located on the same chromosome (chr8) and G2 ancestral block, the CcPYR1 and CcPYL8b genes

also belonged to the same paralogous region (Fig. 2a). A different situation was observed for the CcPYL8a and CcPYL9 genes that derived from different paralogous regions.

Manual curation of CcPYL genes revealed that CcPYR1, CcPYL2, CcPYL7a, CcPYL7b and CcPYL13 did not contain introns, while one intron was found in CcPYL4 and two in CcPYL8a, CcPYL8b and CcPYL9 genes (Fig. 2b). No evidence of 5' UTRs regions was found for CcPYL2, CcPYL7a and CcPYL7b genes. CcPYL8a was the only gene presenting an intron (of 316 bp length) on the 5' UTR region. For CcPYL8b, a 3'UTR extension was based on sequence alignments with a corresponding EST of *C. arabica* (GR997267) expressed in leaf, fruit, flower, root and calli tissues. The CcPYL8b gene was also extended in its 5' UTR using the similarities found in PYL9 genes of tomato (LOC101258886) and potato (LOC102591194) (Table S2). In the same way, an extension was also found in CcPYL13 5'UTR region based on the GT013431 EST sequence of *C. arabica* expressed in fruits. Phylogenetic analyses revealed that the ABA receptors CcPYL8a, CcPYL8b and CcPYL9 belong to the subfamily I together with AtPYL7-10 from *A. thaliana*, CsPYL8-9 from *C. sinensis* and VvPYL8-9 from *V. vinifera* (Fig. 3a, Fig. S2). The CcPYL4 protein was located in the subfamily II together with AtPYL4-6 and CsPYL4-5 while CcPYL13 was closely related to AtPYL11-13. Finally, the subfamily III contained the CcPYR1 and

CcPYL2 proteins, the first being related to the AtPYR1, AtPYL1 and CsPYR1 proteins, and the second to AtPYL2-3 and CsPYL2 proteins.

Phosphatase type 2C (PP2C) proteins

The majority of coffee PP2Cs were identical to phosphatases proteins from *Solanaceae* (Table S3). Among them, the CcABI1, CcABI2, CcHAB and CcHAI were highly similar to potato proteins while CcABI2, CcAHG3 and CcHAB were related to tomato sequences. On the other hand, the CcAHG2 protein presented 57% of identity with a grape sequence. The catalytic domain of PP2Cs composed of 11 conserved motifs with Mg^{2+}/Mn^{2+} [xxD] and [DG] (D: aspartic acid, G: glycine) motifs, was highly conserved throughout the six coffee PP2Cs (Fig. 1b).

Regarding genome localization, these proteins evolved from G2 (*CcAHG2*), G4 (*CcHAB*, *CcABI1*, *CcABI2*) and G6 (*CcHAI* and *CcAHG3*) ancestral blocks (Fig. 2a). Even thought *CcHAB*, *CcABI1* and *CcABI2* in one hand, and *CcHAI* and *CcAHG3* in another, evolved from the same ancestral block genes, all these genes belonged to a different paralogous region. *CcAHG2* and *CcAHG3* contained three introns while four were observed in *CcABI1*, *CcABI2*, *CcHAI* and *CcHAB* genes (Fig. 2b). Only *CcABI1* and *CcABI2* genes contained introns in their corresponding 5'UTR regions.

The phylogenetic analyses revealed that CcABI1-2 and CcHAB proteins evolved together with AtABI1-2, AtHAB1-2, CsABI1 and CsHAB1 while CcAHG2, CcAHG3 and CcHAI were grouped with AtAHG1, AtAHG3, AtHAI1-3 and CsAHG3 (Fig. 3b). The members of the ABA-hypersensitive germination (AHG) subfamily in *C. canephora* were represented by *CcAHG2* and *CcAHG3* genes. Because *CcAHG2* had no ortholog in *A. thaliana* and presented low homology with *AtAHG1*, *AtAHG3*, *AtHAI1-3* and *AtABI1-2* genes, it clustered separately from these genes (Fig.3b, Fig. S3). On the other hand, *CcAHG3* was orthologous to *AtAHG3*, *CsAHG3* and *VvPP2C8*. Finally, the coffee *CcHAI* appeared homologous to ABA-induced genes *AtHAI1*, *AtHAI2* and *AtHAI3*.

SNF1-related (SnRK2) protein kinases

Nine putative SnRK2 protein kinases were identified in *C. canephora*. CcSnRK2.1, CcSnRK2.8, CcSnRK2.12 and CcSnRK2.13 shared high identity (84%, 83%, 92% and 84%) with their respective proteins of *C. sinensis* (Table S4). On the other hand, CcSnRK2.2, CcSnRK2.6 and CcSnRK2.11 had 86%, 93% and 83% of identity with tomato proteins while CcSnRK2.2 and CcSnRK2.10 proteins shared 86% and 91% with potato relatives. Excepted CcSnRK2.12 and CcSnRK2.13, all other coffee SnRK2s contained in their N-terminal region the GXGXXG kinase (ATP binding) domain and the highly acidic ABA box

domain (motif I) important for their interactions with PP2Cs in their C-terminal region (Fig. 1c). In addition to these domains, CcSnRK2.2 and CcSnRK2.6 also contained the C-terminal domains I and II (Fig. 1d) responsible of SnRK2 activation by osmotic stress in ABA-independent and ABA-dependent manners, respectively (Yoshida et al., 2006).

At the gene level, *CcSnRK2.1* had orthologous genes in tomato (*SlSnRK2.1*), grape (*VviSnRK2.12*), and Arabidopsis (*AtSnRK2.1* and *AtSnRK2*.5). On the other hand, *CcSnRK2.10* was orthologous to *VvSnRK2.11* and homologous to *AtSnRK2.10* and *AtSnRK2.4* of Arabidopsis. The *CcSnRK2.7* had an ortholog in *Arabidopsis (AtSnRK2.7*), a co-ortholog in tomato (*SlSnRK2C*) and two homologs in grape (*VvSnRK2.7a* and *VvSnRK2.7b*). *CcSnRK2.8* had two orthologs in Arabidopsis (*AtSnRK2.8*) and grape (*VviSnRK2.8*). According to the classification of Kobayashi *et al.* (2004), *CcSnRK2s* were divided into three subclasses which differed by their activation in response to ABA (Fig. 3c). The *CcSnRK2.1* and *CcSnRK2.10* clustered in the subclass I corresponding to genes not activated in the presence of ABA. The *CcSnRK2.7* and *CcSnRK2.8* belong to the subclass II activated to a lesser extent by ABA. Finally, the subclass III was composed by *CcSnRK2.2* and *CcSnRK2.6* genes strongly activated by ABA. Interestingly, the coffee *CcSnRK2.11* gene did not cluster in any of these subclasses (Fig. S4).

Concerning genome localization, *CcSnRK2.11* gene belong to the G2 ancestral block while *CcSnRK2.2, CcSnRK2.6 CcSnRK2.8, CcSnRK2.7* and *CcSnRK2.10* genes were identified on the G3 block (Fig. 2a). Among them, *CcSnRK2.8* and *CcSnRK2.10*, as well as *CcSnRK2.2* and *CcSnRK2.7* genes, evolved from the same ancestral block and paralogous regions. Excepted *CcSnRK2.12* and *CcSnRK2.13* genes that contained four and five introns, respectively, other *SnRK2* genes contained eight introns (Fig. 2b). Because *CcSnRK2.12* and *CcSnRK2.13* genes also missed a stop codon, they were considered as uncompleted sequences and were not further analyzed.

Expression profiles of CcPYLs-PP2Cs-SnRK2 genes in leaves and roots of C. canephora submitted to drought conditions

Expression of PYL/PYR/RCAR-PP2C-SnRK2 genes was analyzed in leaves and roots of the D^T and D^S clones of *C. canephora* grown under I (irrigated) and NI (non-irrigated) (Fig. 4). Whatever the primer pairs designed for *CcPYL7a*, *CcPYL7b* and *CcPYL13* (Table S1) and irrigation conditions, no expression was detected in leaves and roots (data not shown). For *CcPYR1* and *CcPYL4*, expression was observed in leaves of all clones under control condition and decreased under drought. A similar pattern was observed for *CcPYL2*, except that this gene was expressed under irrigation only in leaves in D^T clones 14 and 120. Expression levels of *CcPYL8a* gene did not changed significantly from I to NI conditions in leaves of D^T clones 14, 73 and 120 but decreased significantly under

drought in D^S clone 22. Whatever the clones, *CcPYL8b* and *CcPYL9* were the most expressed genes in leaves of *C. canephora* plants under irrigation. However, expression of *CcPYL8b* and *CcPYL9* genes increased significantly under drought in leaves of clones 22, 73 and 120, and in those of D^T clones 14 and 73, respectively.

In roots, expression of *CcPYR1*, and *CcPYL4* decreased under drought in all clones of *C. canephora* (Fig. 5). On the other hand, *CcPYL8b* gene expression was significantly induced by drought in the D^T clones 73 and 120. Up-regulated expression of *CcPYL9* was also noticed in D^T clone 73 under drought.

For the *PP2C* genes, expression of *CcABI2*, *CcAHG3* and *CcHAI* genes were significantly up-regulated under drought in leaves and roots of both D^T and D^S clones (Fig. 4). Drought-induced expression of*CcAHG2* and *CcHAB* was also observed but only in leaves of the D^T clones 14, 73 and 120. Different expression profiles were observed for *CcABI1* in leaves and roots with water conditions. For example, *CcABI1* expression was up-regulated under drought only in D^T clone 73, but down-regulated by drought in D^S clone 22. On the other hand, while *CcABI1* gene expression decreased under drought in the D^T clone 14, it was highly up-regulated by drought in the D^T clone 120. Compared to other *PP2Cs*, *CcHAI* was the most expressed in both leaves and roots under drought. Expression of *CcAHG2* was also greatly up-regulated by drought in leaves of all D^T clones but not in those of D^S clone 22. However, no detectable expression of this gene was

observed in roots (data not shown). In roots, the D^T clone 120 stands out other clones by the fact that it presented high up-regulated expression under drought of *CcABI1*, *CcABI2* and *CcAHG3*, as well as of *CcSnRK2.2*, *CcSnRK2.6* and *CcSnRK2.8* genes. The expression of subclass III *CcSnRK2.2* gene was also up-regulated by drought in leaves of the D^T clones 14 and 73 but also in roots of all D^T clones. On the other hand, *CcSnRK2.6* gene expression was unaffected by water condition in leaves but increased under NI conditions only in roots of D^T clone 73.

The expression of *CcSnRK2.7* gene increased under NI conditions in leaves in the D^T clone 73 and mainly in roots of D^T clone 120 (Fig. 4). On the other hand, expression of *CcSnRK2.8* was up-regulated by drought only in leaves of D^T clone 73 as well as in roots of D^T clone 73 and D^S clone 22. No significant differences of expression profiles were observed for *CcSnRK2.10* and *CcSnRK2.11* in leaves of all coffee clones. However, *CcSnRK2.10* gene expression was down-regulated under drought in roots of D^T clones 14 and 120, but unaffected in clones 73 and 22. While *CcSnRK2.11* expression was not detected in roots (data not shown), expression levels detected in leaves were not significantly affected by water treatments. For *CcSnRK2.1*, as well as *CcSnRK2.12* and *CcSnRK2.13*, expression was undetectable in drought-stressed roots and leaves of all coffee clones with the tested primer pairs (data not shown).

ABA quantification

In leaves, ABA was detected in all clones under both I and NI conditions, ranging from 2 to 8 pmol.g^{-1} of DW (Fig. 5). A significant increase of ABA content under drought was observed in leaves of D^T clone 120 while ABA contents were considered as relatively stable in other clones whatever the irrigation conditions. In roots, ABA contents were similar (around 4 pmol.g^{-1} of DW) in all clones under irrigated conditions. If these contents tended to decrease under drought in all clones, this reduction was significant only in roots of the D^T clone 14.

DISCUSSION

For the first time, the orthologous genes coding for proteins of the PYL/PYR/RCAR-PP2C-SnRK2 tripartite system involved in the first steps of ABA perception and signal transduction were identified and thoroughly characterized in *C. canephora*. Based on sequence similarity with other plant genes, nine *CcPYL*-type genes, six *PP2C*-type and nine *SnRK2*-type genes divided in three subclasses were found.

PYR/PYL/RCAR gene family in C. canephora

Nine *PYR/PYL/RCAR* genes were found in the *C. canephora* genome. This number was similar to *PYLs* found in *C. sinensis* (Romero et al., 2012) and

446 *V. vinifera* (Boneh et al., 2012b), but smaller than *PYLs* in Arabidopsis (Ma et al.,
447 2009; Park et al., 2009), tomato (Gonzalez-Guzman et al., 2014) and rice (Kim et
448 al., 2012). Interestingly, *C. canephora* contained duplicated genes of *PYL7*
449 (*CcPYL7a* and *CcPYL7b*) and *PYL8* (*CcPYL8a* and *CcPYL8b*) (Fig. 6). The
450 duplicated *CcPYL7s* were located into the chr0 corresponding to unmapped
451 scaffolds grouped arbitrary in a pseudomolecule (Denoeud et al., 2014) and not
452 expressed in leaves or roots of *C. canephora*. These results are in accordance with
453 *in silico* data deduced from the Coffee Genome Database (Fig. S1). However,
454 since *CcPYL7a* and *CcPYL7b* were expressed in developing beans of *C. arabica*
455 (data not shown), it can be concluded that they correspond to functional genes like
456 *CcPYL8a* (chr2) and *CcPYL8b* (chr8).

457 Denoeud *et al.* (2014) recently reported that the coffee genome contained
458 several species-specific gene families that probably occurred by segmental and
459 tandem gene duplication, as well as transposition events. Despite the fact that
460 *CcPYL8a*, *CcPYL8b* and *CcPYL9* harboured different chromosome localizations,
461 their chromosome position, origin, similar gene structure and expression profiles
462 suggested that they underwent duplications (Fig. 6). This hypothesis is supported
463 by the fact that these genomic fragments harboured other duplicated genes (e.g.
464 lipid transfer protein, zinc finger DOF protein, heat shock protein, Dehydration-
465 responsive element-binding protein 1D) (data not shown) previously shown to be

important in responses of *C. canephora* (Marraccini et al., 2012; Vieira et al., 2013) and *C. arabica* (Mofatto et al., 2016) to drought.

In the present work, *CcPYL8a* and *CcPYL8b* paralogs showed different expression profiles in roots under drought. Such differences could be explained by the presence of the 316 bp intron in the 5' UTR region of *CcPYL8a* affecting expression of this gene. This hypothesis is reinforced by the presence of two LTR *copia* retrotransposons in *CcPYL8a*, one located in its promoter region (2 kb) and the other in its first intron. TEs located near host genes are known to impact gene expression and to play a role in the genome adaptation to environmental changes (Casacuberta & González, 2013), as suggested in coffee where high TEs expression was observed in *C. canephora* and *C. arabica* submitted to drought (Lopes et al., 2013).

To our knowledge, the results presented here are the first reporting functional duplication of *PYL8* gene. They demonstrated that *CcPYL8b* and *CcPYL9* were the genes mostly expressed in roots and leaves of *C. canephora* indicating their probable key role to cope with drought in coffee, as also suggested in Arabidopsis (Ma et al., 2009; Zhao et al., 2014). However, expression of *CcPYL8a, CcPYR1, CcPYL2* and *CcPYL4* was unaffected by drought, suggesting that these genes played a limited role in the response of *C. canephora* to water limitation.

Coffee PP2C gene family

Six *CcPP2Cs* were identified in the *C. canephora* genome. This gene number is higher to that found in *C. sinensis* (Romero et al., 2012), but lower to that of Arabidopsis (Ma et al., 2009; Park et al., 2009), grape (Boneh et al., 2012a), tomato (Sun et al., 2011) and rice (Xue et al., 2008). Expression analyses revealed that these coffee genes were functional since they were all expressed in leaves particularly in drought stressed coffee. Among them, *CcHAI* retained attention since its expression was low under unstressed conditions but highly induced under drought in all clones. In roots, this gene was highly up-regulated under drought in D^T clone 73 and D^S clone 22, while the increase was much more reduced in D^T clones 14 and 120. In Arabidopsis, *hai* mutants exhibited inhibition of root growth and induction of many ABA-regulated genes such as dehydrins, late embryogenesis abundant proteins, *NCED3* and *NACs* (Bhaskara et al., 2012). Here, *CcHAI* was the gene mostly up-regulated under drought in leaves and roots of all *C. canephora* clones, suggesting its key role in coffee responses to drought.

Several studies already reported induced expression of the *PP2C* genes under abiotic stress (Tähtiharju & Palva, 2001), as observed for *ABI1*, *ABI2* and *HAB1* in leaves of Arabidopsis early during drought treatment (Harb et al., 2010). *ABI1* is a key gene of ABA signaling in the guard cells where ABI1 inhibition after ABA perception stimulates stomatal closure (Saez et al., 2006). Such a role is not expected in roots where expression of *CcABI1* and *CcABI2* was highly up-

regulated under drought, particularly in D^T clone 120. The fact that these two genes exhibited similar expression profiles could be explained by their overlapping roles in controlling ABA action (Leung et al., 1997; Merlot et al., 2001). The up-regulated expression of *CcAHG3* also observed in parallel to the accumulation of *CcABI1* and *CcABI2* transcripts might be related with the function of this gene in ABA response pathway (Nishimura et al., 2004). As previous studies shown that high concentrations of ABA inhibit root growth (Beaudoin et al., 2000), it is possible that these *PP2C* genes could act together on the development of coffee root system under drought.

Another interesting result concerned *CcAHG2* whose expression was significantly up-regulated in leaves under drought specifically in D^T clones and undetected in roots. These expression profiles are not contradictory to those of *in silico* (Fig. S1) that did not detect *CcAHG2* expression in leaves since RNA-seq libraries were generated from unstressed coffee plants (Dereeper et al., 2015). The fact that *CcAHG2* was expressed in drought-stressed leaves of D^T clones but not in those of D^S clone 22, highly suggests a key function of this gene in leaves of *C. canephora* D^T clones submitted drought.

SnRK2 gene family in C. canephora

In this work, nine putative *SnRK2* genes were identified in the *C. canephora* genome. Expression studies revealed that *SnRK2.2* was up-regulated

upon drought in leaves of D^T clones 14 and 73. For other *SnRK2* genes, expression levels can be considered as relatively stable and poorly affected by drought in all *C. canephora* clones. An opposite situation was observed in roots in which the expression profiles of *CcSnRK2.2*, *CcSnRK2.6* and *CcSnRK2.7* genes were highly up-regulated upon drought, mainly in D^T clone 120.

Among SnRK2 proteins, those of subgroup III (e.g. *SnRK2.2* and *SnRK2.6*) play important roles in ABA-induced stomatal closure (Cutler et al., 2010). Phosphorylated forms of SnRK2.2 and 2.6 were also reported to activate the ABA-responsive structural gene *RD29B* (Yoshida et al., 2010). Zheng *et al.* (2010) also reported the role of *SnRK2.6* in increasing carbon supply and stimulating plant growth. Even though some functional redundancy had been postulated between SnRK2.2 and SnRK2.6 (Fujii & Zhu, 2009), our results clearly suggest a key role of these kinases in response to drought, mainly in roots of *C. canephora*.

In contrast to subgroup III, the main targets of subgroup II SnRK2s are stress-responsive genes coding transcription factors (Kulik et al., 2011). For example, Zhang *et al.* (2010) showed that over-expression of wheat *SnRK2.8* in Arabidopsis enhanced tolerance to drought, salt and cold stresses by up-regulating the expression of genes involved in ABA biosynthesis and signaling. On the other hand, *A. thaliana* over-expressing *SnRK2.7* from wheat showed enhanced photosystem II activity and root growth (Zhang et al., 2011). Even though

SnRK2.7 and SnRK2.8 might be functionally redundant, *SnRK2*.7 was shown to be expressed in roots, leaves and flowers of Arabidopsis while *SnRK2.8* was mainly expressed in roots, indicating different tissue specificities of these two kinases (Mizoguchi et al., 2010). The up-regulated expression of *CcSnRK2*.7 and *CcSnRK2.8* in roots of drought-stressed *C. canephora*, led us to propose key functions of both kinases in coffee roots.

How the tripartite system PYL-PP2C-SNRK2 of ABA perception could explain D^T and D^S phenotypes of C. canephora clones?

In higher plants, ABA content is rigorously controlled by the rate of biosynthesis, catabolism, compartmentalization and transport, increasing in both roots and leaves in response to water deficit. Here, we showed that D^T clone 120 was the only one presenting significant increase of leaf ABA content under drought. Whatever the *C. canephora* clone, no significant differences of ABA contents were observed in roots, therefore indicating that D^T and D^S phenotypes were probably due to altered ABA signalling pathway rather than deficiencies of ABA synthesis.

Previous studies revealed that transport rate/CO_2 assimilation (*ETR*/*A*) ratio was significantly higher under drought in D^T clone 73 compared to D^T clones 14 and 120, therefore suggesting the participation of an alternative electron sink protecting the photosynthetic apparatus against photoinhibition by limiting electron accumulation and ROS formation in clone 73. Interestingly, drought-

induced up-regulated expression of genes encoding for ascorbate peroxidase (*CcAPX1*), a prephenate-dehydrogenase like protein (*CcPDH1*) and a non-symbiotic haemoglobin (*CcNSH1*) was already reported in this clone, suggesting its protection involved strong induction of antioxidant and osmoprotection systems (Vieira et al., 2013). The up-regulated expression of *SnRK2.2*, *SnRK2.7* and *SnRK2.8* upon drought in its leaves could participate in activating such pathways.

Another interesting result concerned *CcAHG2* that was expressed only in leaves of all D^T clones but not in those of D^S clone 22. Because *CcAHG2* lacks ortholog in *A. thaliana*, its biochemical function is unknown. Further research is therefore needed to know if *CcAHG2* could be used as a molecular marker of drought tolerance in coffee.

Compared to D^S clones of *C. canephora*, it was already reported that D^T clone 120 had a deeper root system that should allow greater access to soil water (Pinheiro et al., 2005). Interestingly, *CcPYL8b*, but also of *SnRK2* (*CcSnRK2.2*, *CcSnRK2.6* and *CcSnRK2.7*) and *PP2C* (*CcABI1*, *CcABI2* and *CcAHG3*) genes were highly up-regulated under drought in roots, indicating a key role of root system in responses to drought in this clone. Even though D^T clone 14 also had a root depth similar to clone 120 (Pinheiro et al., 2005), it did not showed up-regulate expression of *PYL*, *SnRK2* and *PP2C* genes in roots, indicating the existence of different mechanisms amongst the D^T coffee clones regarding water

deficit (Vieira et al., 2013). Whatever it is, the differences observed for *SnRK2* (mainly of subclass III) gene expression profiles clearly indicated the involvement of the ABA-dependent signalling pathway in the response to drought, at least in D^T clones. Of course, this does not preclude the involvement of other hormonal regulatory pathways in the establisment of drought tolerance phenotypes in coffee. For example, up-regulated expression of subclass II *SnRK2* genes by salicylic acid, ethylene, and jasmonates, has already been reported (Kulik et al., 2011). The occurence of such entangled crosstalks between biotic and abiotic pathways might exist in coffee, as suggested by the fact that the D^T clone 14 was also recently identified as resistant to multiple races of root-knot nematodes *Meloidogyne* (Lima et al., 2015).

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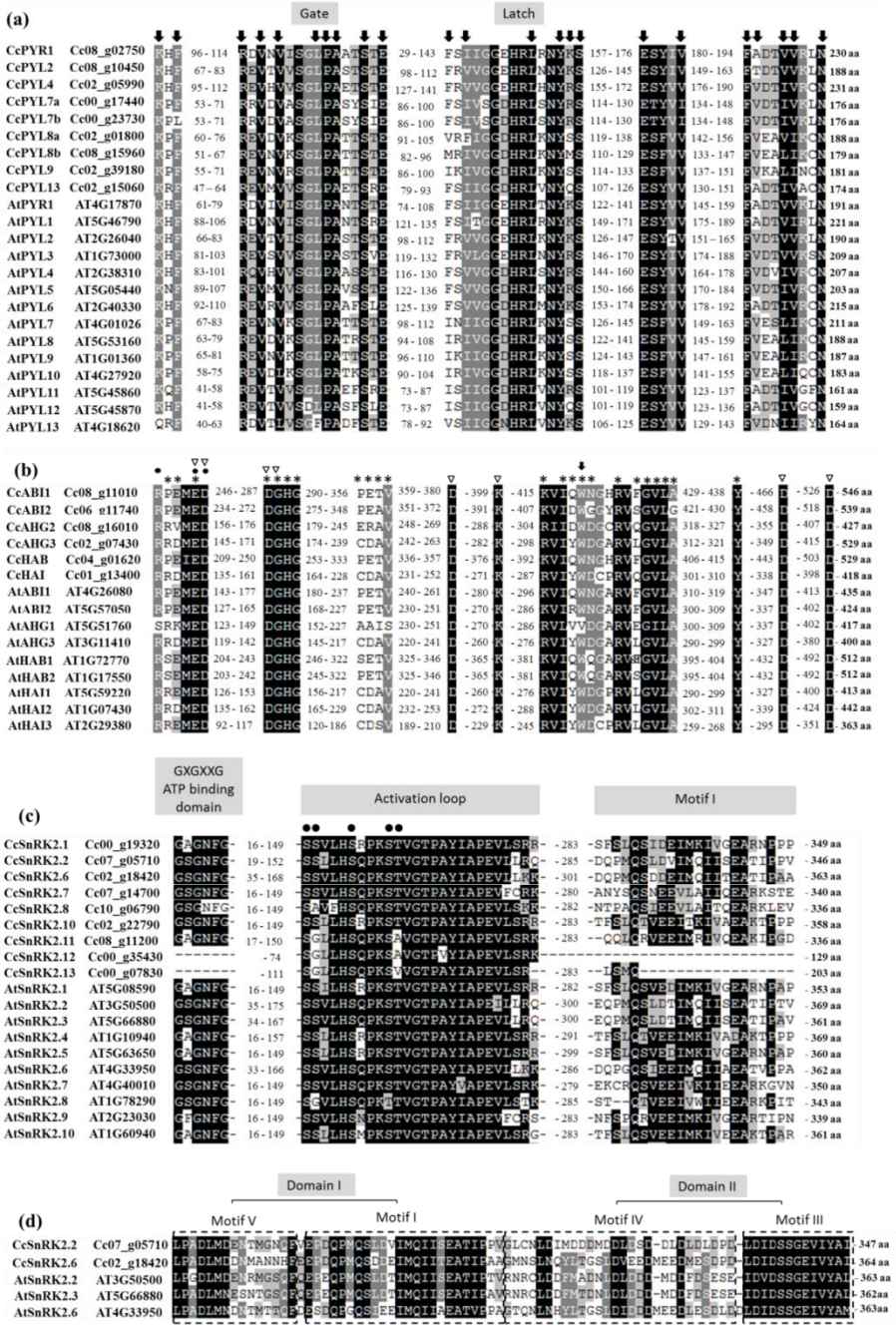

Figure 1 Sequence alignments of the PYL, PP2C and SnRK2 putative proteins (…continue…)

Amino acid sequences are shown only for functional residues and conserved domains. For each protein, total length is indicated in amino acids (aa). Conserved residues are marked with black or grey shading. (a): sequence alignment of the PYL proteins. Residues forming the ligand-binding pocket are marked by black arrows. The gate and latch domains are indicated. (b): sequence alignment of the PP2C proteins. Residues interacting with ABA, PYLs and Mn^{2+}/Mg^{2+} ions are marked by black arrows, asterisks, and white triangles, respectively. Phosphatase sites are marked with black points. (c): sequence alignment of C-terminal regions of subclass III SnRK2s. Functional domains (ATP binding site, activation loop and motif I) are indicated. (d): sequence alignment of C-terminal regions of subclass III SnRK2s. Functional domains (domains I and II with their corresponding motifs) are indicated.

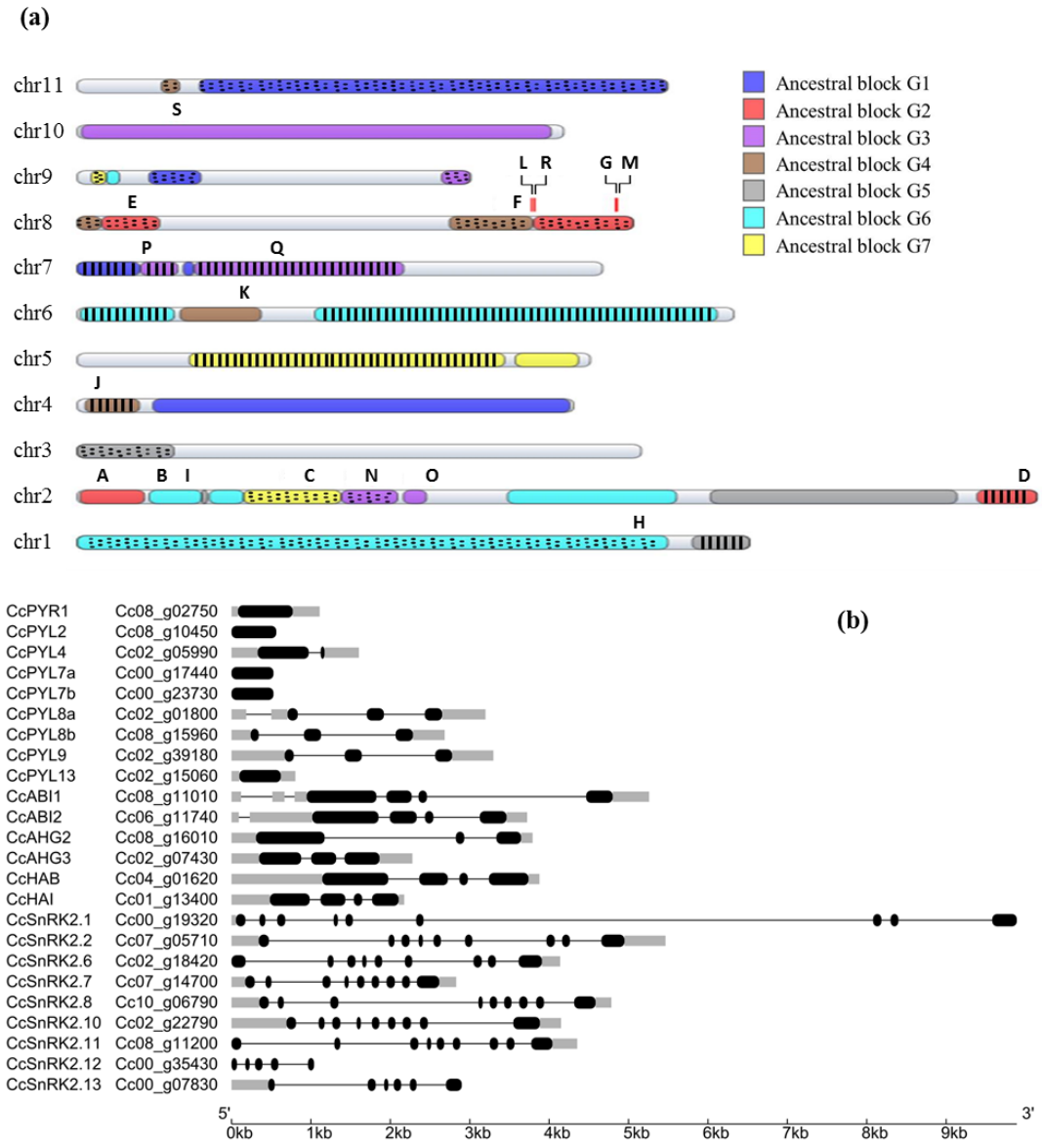

Figure 2 Localization and structure of *PYR/PYL/RCAR*, *PP2C* and *SnRK2* genes (…continue…)

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(a): localization of genes in *C. canephora* chromosomes. *CcPYL* genes: *CcPYL8a* (A), *CcPYL4* (B), *CcPYL13* (C), *CcPYL9* (D), *CcPYR1* (E), *CcPYL2* (F) and *CcPYL8b* (G). *CcPP2C* genes: *CcHAI* (H), *CcAHG3* (I), *CcHAB* (J), *CcABI2* (K), *CcABI1* (L) and *CcAHG2* (M). *CcSnRK2* genes: *CcSnRK2.6* (N), *CcSnRK2.10* (O), *CcSnRK2.2* (P), *CcSnRK2.7* (Q), *CcSnRK2.11* (R) and *CcSnRK2.8* (S). The *PYLs* (*CcPYL7a* and *CcPYL7b*, and *SnRK2 (CcSnRK2.13)*, *CcSnRK2.1* and *CcSnRK2.12* genes unanchored in the chromosome 0 and are not indicated. The coloured regions represent the ancestral blocks of the 7 core eudicot chromosomes (adapted from Denoeud *et al.* [2014]). (b): structure of *CcPYL*, *CcPP2C* and *CcSnRK2* genes. The black blocks represent exons, the gray blocks the upstream and downstream transcribed and untranslated regions (UTRs) and the lines the introns. The structure of genes located in the chromosome 0 is not represented. For the *CcPYL2*, *CcPYL7a* and *CcPYL7b* genes, no 5' and 3'UTRs were found.

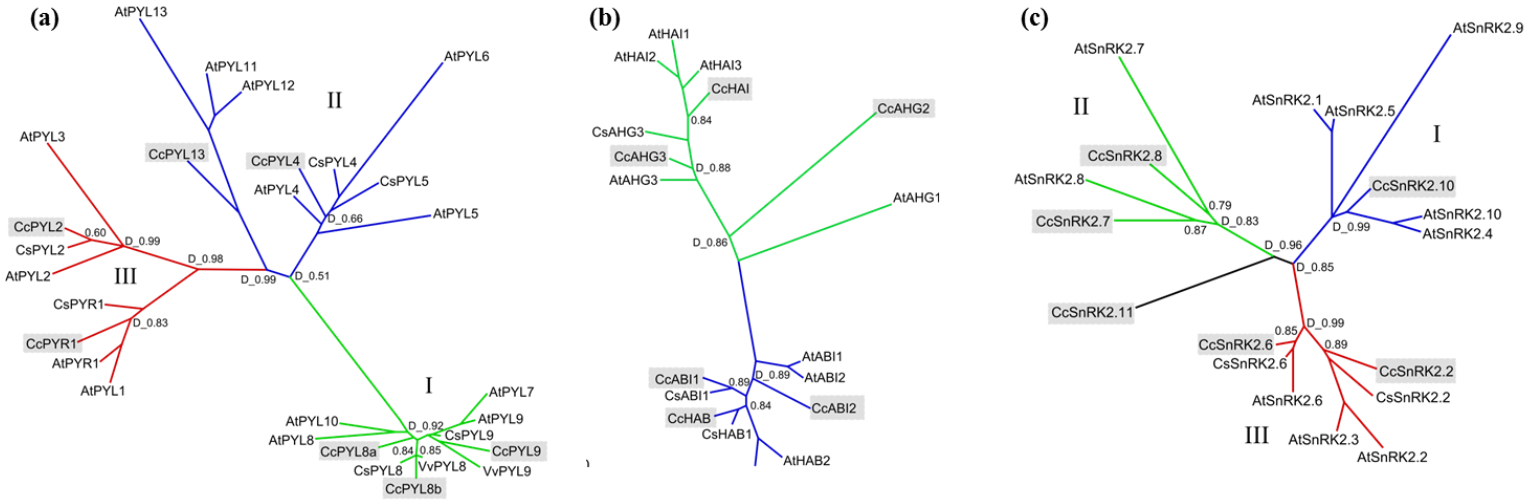


Figure 3 Phylogenetic analyses of *C. canephora* PYR/PYL (a), clade-A PP2C (b) and SnRK2 (c) proteins. Trees were constructed using amino proteins of *C. canephora* and orthologous proteins from *A. thaliana* (At), *C. sinensis* (Cs) and *V. vinifera* (Vv) (see Tables S2-S4 and Fig. S2-S4). The coffee proteins are highlighted in gray. The proteins coded by genes located in the chromosome 0 are not included. For PYR/PYL and SnRK2 trees, protein subclasses are also indicated.

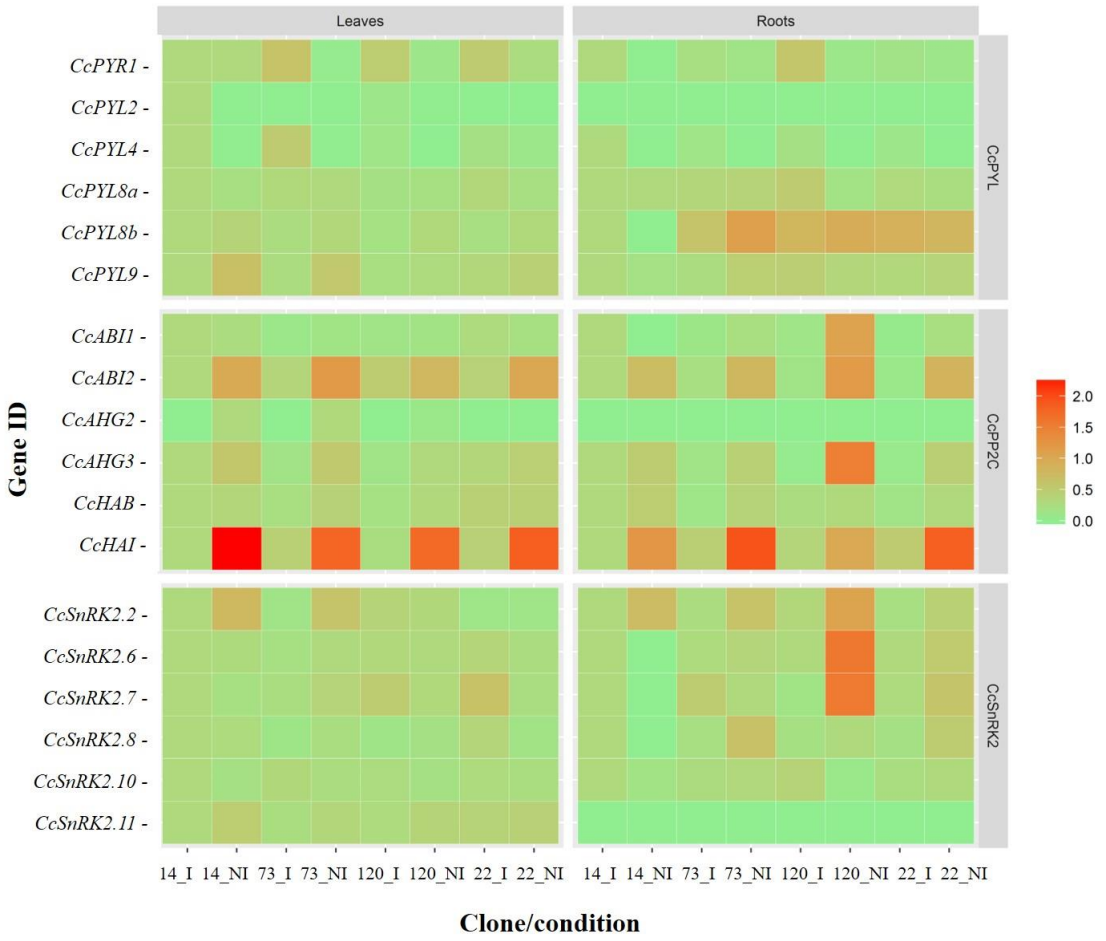


Figure 4 Expression profiles of *PYR/PYL*, *PP2C* and *SnRK2* genes in leaves and roots of D^T (14, 73 and 120) and D^S (22) clones of *C. canephora* subjected (NI) or not (I) to drought. The gene names are indicated in the heatmap. Values are the mean of at least three technical repetitions ± SD which are standardized independently with *CcUBQ10* (ubiquitin) as reference gene. Results are expressed using 14I as an internal calibrator (RE=1), except for *CcAHG2* gene where 14NI was used. Higher expression for each gene was presented in red, otherwise, green was used.

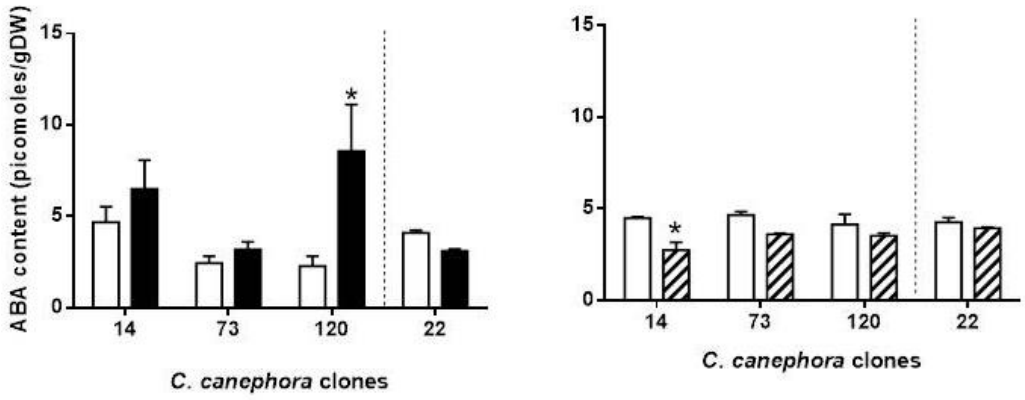


Figure 5 ABA content of leaves and roots of D^T (14, 73 and 120) and D^S (22) clones of *C. canephora* subjected (NI) or not (I: white isobars) to drought. Black and striped isobars corresponded to drought conditions in leaves and roots, respectively**.** For the statistical analysis, significant differences ($P \leq 0.05$) between the treatments were evaluated using 2way ANOVA test (non-parametric test) and are indicated by an asterisk.

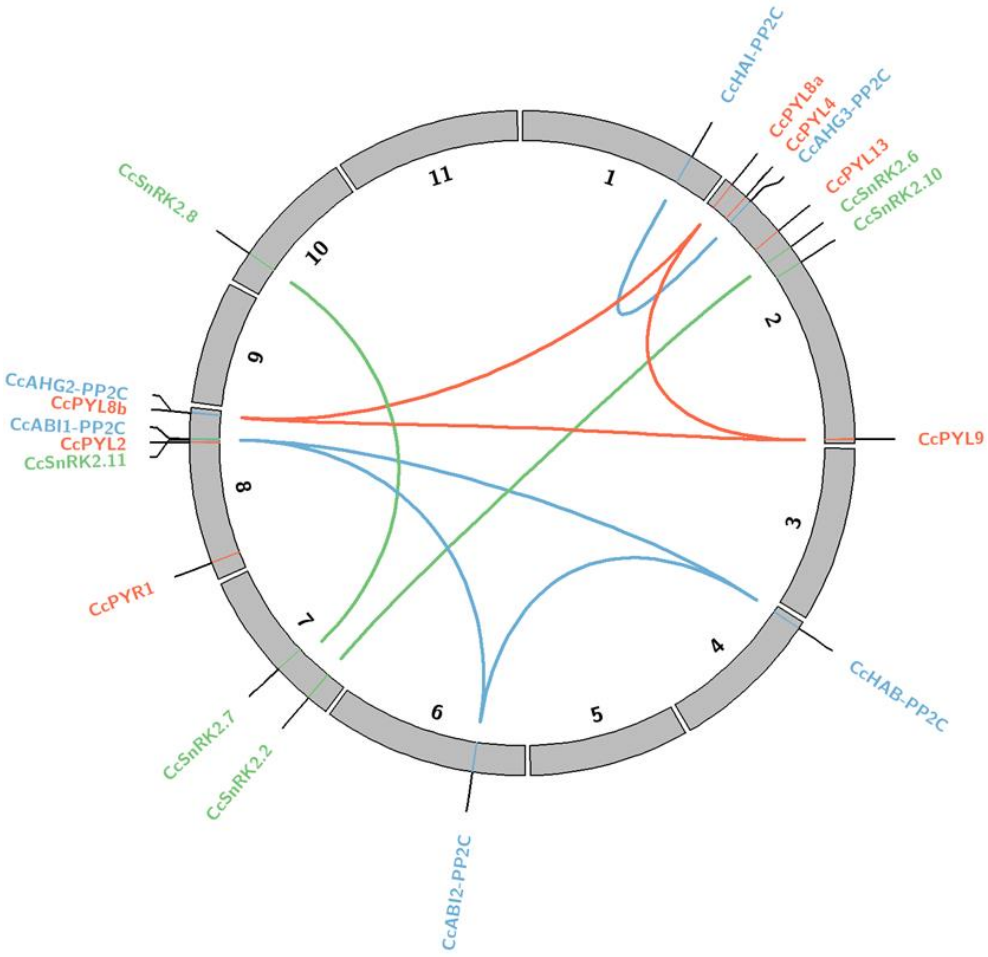
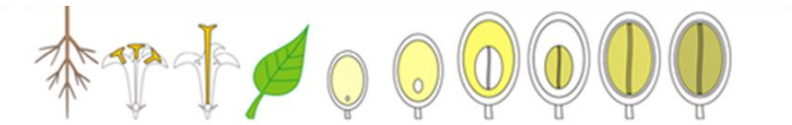


Figure 6 Graphical representation of the *CcPYL-CcPP2C-CcSnRK2* duplicated genes on *C. canephora* chromosomes (indicated by numbers, from 1 to 11). The *CcPYL*, *CcPP2C* and *CcSnRK2* duplications genes are indicated by with red, blue and green lines, respectively. The *CcPYL8a*, *CcPYL8b*, *CcPYL9*, *CcABI1*, *CcABI2* and *CcHAB* as well as *CcSnRK2.2* and *CcSnRK2.6*, evolved through proximal duplications. The genes located on the chromosome 0 are not showed.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:



(a)	***CcPYLs***										
CcPYR1	*Cc08_g02750*	71	2	12	9	1	2	3	9	10	82
CcPYL2	*Cc08_g10450*	0	0	5	0	0	1	1	30	14	6
CcPYL4	*Cc02_g05990*	36	103	61	4	128	171	168	21	16	223
CcPYL7a	*Cc00_g17440*	0	1	0	0	0	0	0	0	0	1
CcPYL7b	*Cc00_g23730*	0	19	0	0	0	0	0	1	0	1
CcPYL8a	*Cc02_g01800*	63	13	56	31	28	30	81	71	54	169
CcPYL8b	*Cc08_g15960*	123	21	71	103	152	93	122	40	61	116
CcPYL9	*Cc02_g39180*	243	28	84	142	169	89	140	41	42	65
CcPYL13	*Cc02_g15060*	0	0	2	0	3	6	18	36	67	129
(b)	***CcPP2Cs***										
CcABI1	*Cc08_g11010*	195	19	46	214	36	37	14	14	24	21
CcABI2	*Cc06_g11740*	112	39	48	100	28	35	22	17	45	62
CcAHG2	*Cc08_g16010*	4	2	1	6	0	1	2	61	82	164
CcAHG3	*Cc02_g07430*	261	74	214	227	17	9	6	82	124	208
CcHAB	*Cc04_g01620*	82	12	63	78	23	23	17	11	32	47
CcHAI	*Cc01_g13400*	103	16	151	156	39	58	40	108	150	135
(c)	***CcSnRK2s***										
CcSnRK2.1	*Cc00_g19320*	0	139	0	0	0	0	0	0	0	0
CcSnRK2.2	*Cc07_g05710*	219	28	16	18	58	44	47	27	25	136
CcSnRK2.6	*Cc02_g18420*	86	16	65	52	33	24	15	12	51	33
CcSnRK2.7	*Cc07_g14700*	85	97	10	93	32	23	18	6	3	2
CcSnRK2.8	*Cc10_g06790*	38	13	9	34	28	15	9	22	37	36
CcSnRK2.10	*Cc02_g22790*	93	38	82	74	80	89	45	70	63	46
CcSnRK2.11	*Cc08_g11200*	12	0	1	2	3	2	2	22	33	41
CcSnRK2.12	*Cc00_g35430*	0	0	2	0	3	2	1	1	2	1
CcSnRK2.13	*Cc00_g07830*	3	3	8	2	11	6	3	4	6	4

Figure S1 Heatmap visualization of the *CcPYR/PYL* (a), *CcPP2C* (b) and *CcSnRK2* (c) gene families (…continue…)

From left to right, the libraries correspond to root, stamen, pistil, leaf, perisperm (120, 150 and 180 days after pollination-DAP) and endosperm (180, 260 and 320 DAP) from *C. canephora* RNA-Seq data. Transcript abundance was normalized with RPKM and the level of gene expression is indicated with a colour scale, from white (weakly expressed) to red (strongly expressed) (adapted from: http://www.coffee-genome.org/).

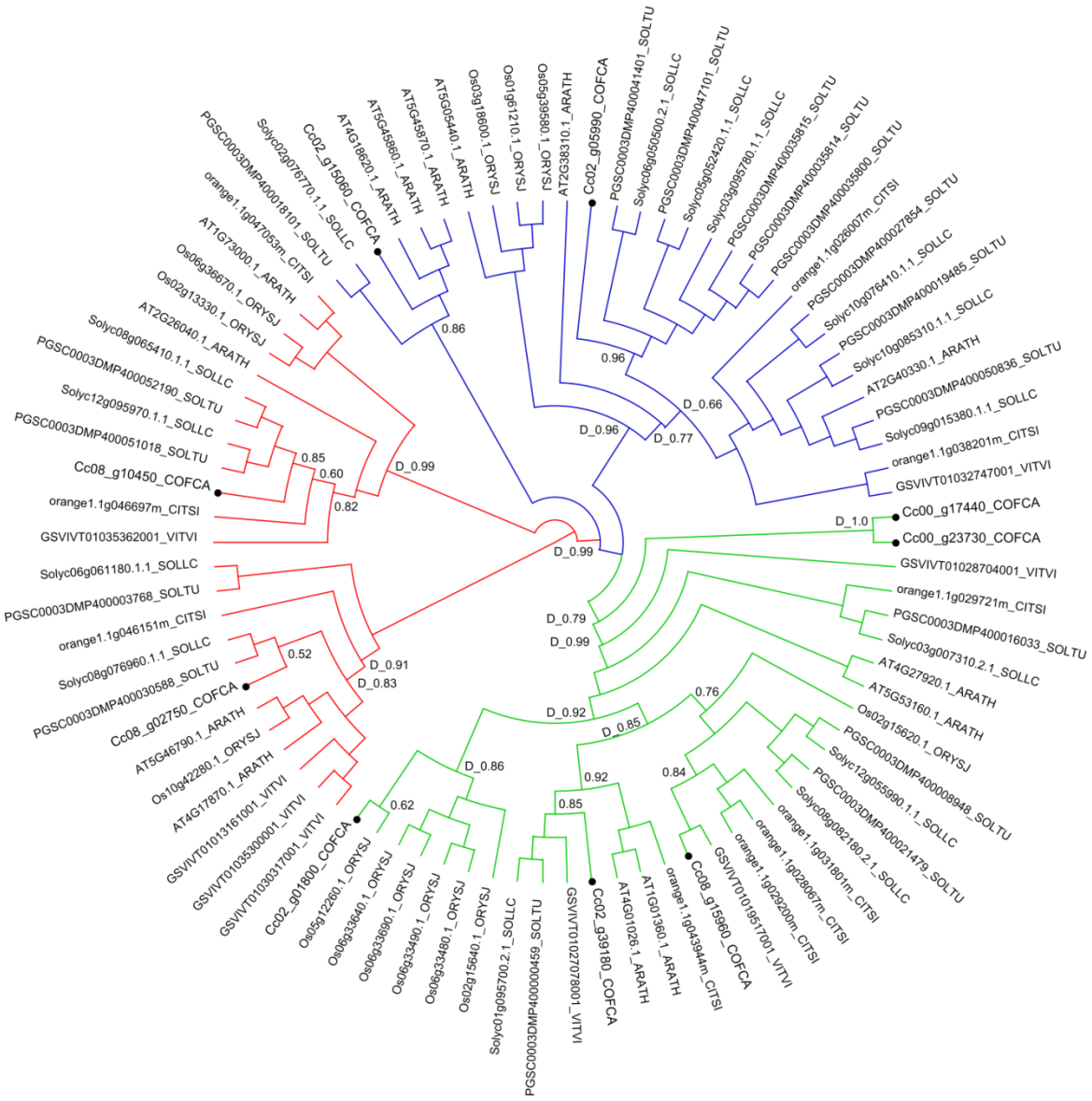


Figure S2 Phylogenetic analysis of CcPYL protein sequences with orthologous proteins of *A. thaliana* (ARATH), *C. sinensis* (CITSI), *O. sativa* (ORYSJ), *S. lycopersicum* (SOLLC), *S. tuberosum* (SOLTU) and *V. vinifera* (VITVI). The phylo-HMM approach was based on NNI (Nearest Neighbor Interchange) topology. Subfamilies I (green), II (blue) and III (red) are indicated. Main bootstraps values are indicated.

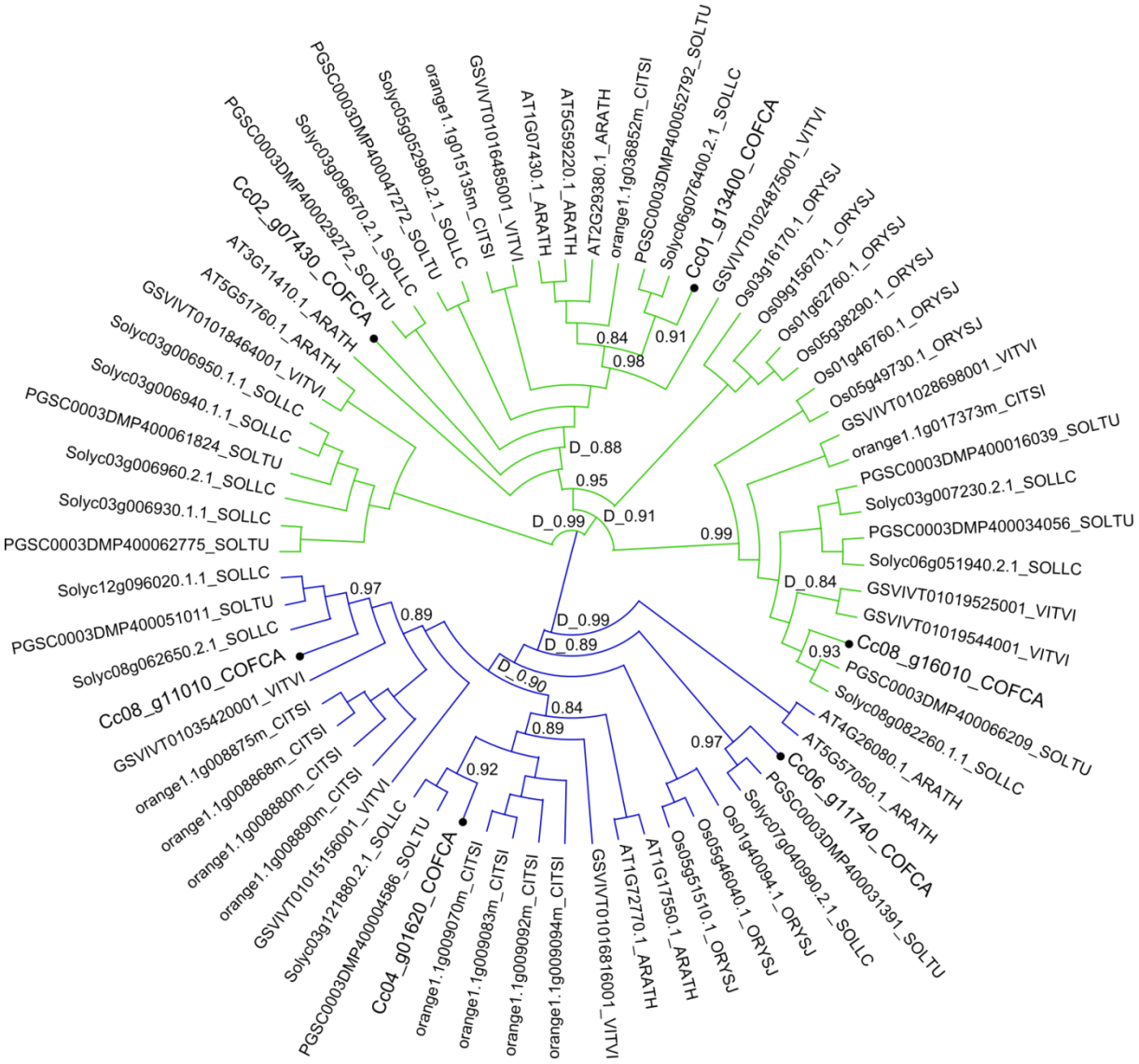


Figure S3 Phylogenetic analyses of PP2C protein sequences with orthologous proteins of *A. thaliana* (ARATH), *C. sinensis* (CITSI), *O. sativa* (ORYSJ), *S. lycopersicum* (SOLLC), *S. tuberosum* (SOLTU) *and V. vinifera* (VITVI). The phylo-HMM approach was based on NNI (Nearest Neighbor Interchange) topology. Subfamilies are indicated with different colours. Main bootstraps values are indicated.

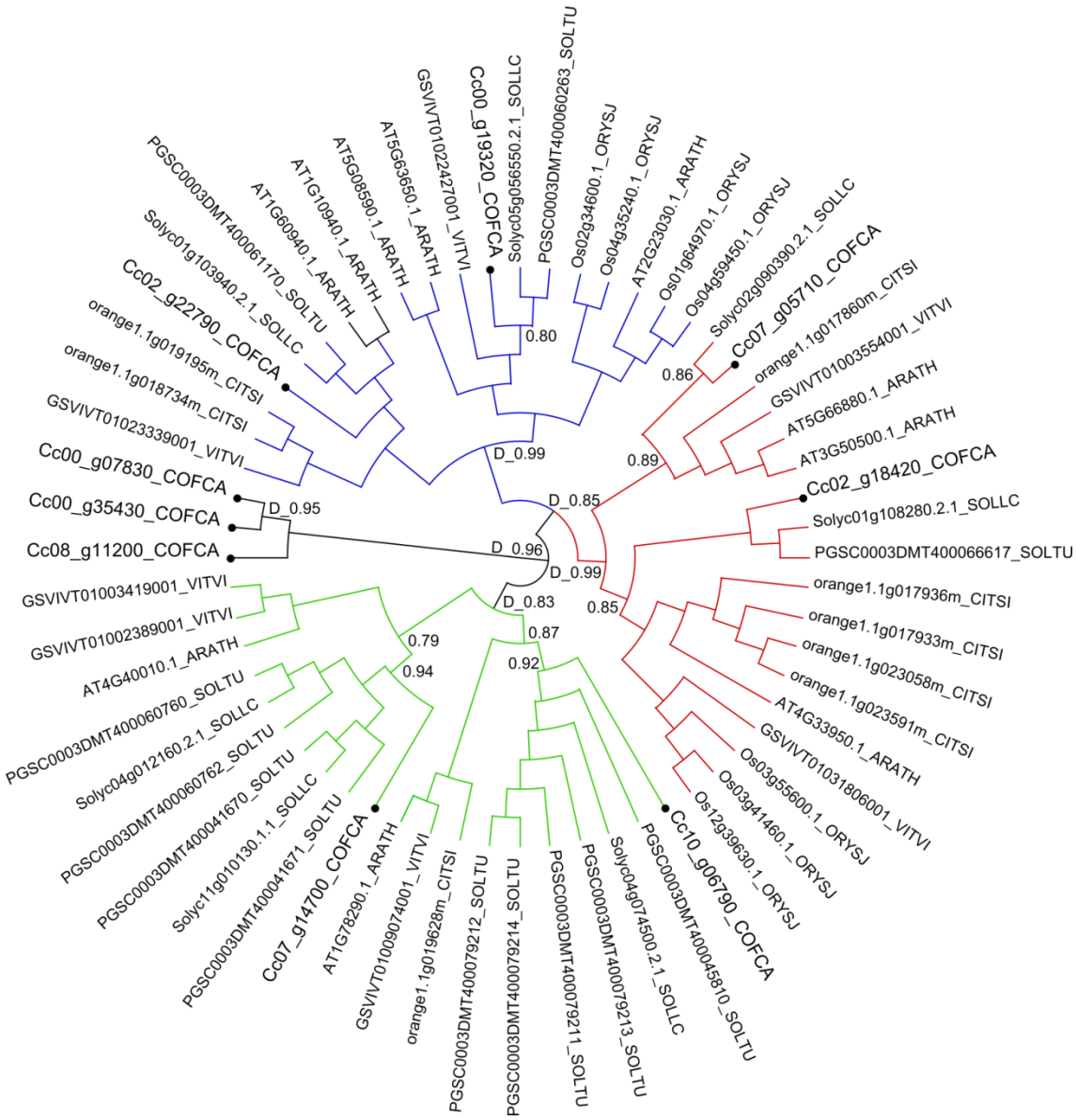


Figure S4 Phylogenetic analyses of SnRK2 protein sequences with orthologous proteins of *A. thaliana* (ARATH), *C. sinensis* (CITSI), *O. sativa* (ORYSJ), *S. lycopersicum* (SOLLC), *S. tuberosum* (SOLTU) *and V. vinifera* (VITVI). The phylo-HMM approach was based on NNI (Nearest Neighbor Interchange) topology. Subfamilies I (green), II (blue) and III (red) are indicated. Main bootstraps values are indicated.

Table 1 Candidate genes and corresponding primers used for qPCR experiments. Pairs of primers were designed for each gene using the Primer Express software (Applied Biosystems). The primers select to qPCR experiments F (Forward) and R (Reverse) are indicated. For *Cc02_g05990* and *Cc10_g06790* genes two different pairs of primers were used in each tissue, F1R1 (leaves) and F2R2 (roots). The *CcUBQ10*F and R primer par was used for the ubiquitin (UBI) as reference gene.

Gene Family	Gene Name	Gene ID	Primer sequence 5'-3'
PYR/PYL/RCAR	*CcPYR1*	*Cc08_g02750*	F: CGGTGACGACTGTCCATGAG R: TCCGGCACGTCAACGATATA
	CcPYL2	*Cc08_g10450*	F: AAAAGTGGTGTGGCCATTCG R: CTTCCATCCCCTGTCATGTTG
	CcPYL4	*Cc02_g05990*	F1: CCTATGCCTTCGTCCCTTCA R1: CGCGGAATTGGTGGTTGTAG F2: TACCATTGTGCGGTGCAACT R2: TTCTGTTCTGGGCTTCCATGA
	CcPYL7a	*Cc00_g17400*	F: GAGCGGCTCGAGACTCTTGA R: GCCGCTGACAATGCTGAAC
	CcPYL7b	*Cc00_g23730*	F: GCGGCTGGAGATTCTTGATC R: CGCCTGGCCATCTATGATTC
	CcPYL8a	*Cc02_g01800*	F: GGTTTGATCAGCCCCAGAAAT R: CCACTTCCCTAAGGCTTCCAA
	CcPYL8b	*Cc08_g15960*	F: GCCAGAGGGAAATACCAAGGA R: CAGCTAGGCGCTCTGAGACA
	CcPYL9	*Cc02_g39180*	F: CACCCGTGCTCTTCCTCTGT R: TCCTCACCAGTGACCAAACG
	CcPYL13	*Cc02_g15060*	F: TCCCAAACCAGTGCACTTCA R: TTGTCGAATTGACGGACCAA
PP2C	*CcABI1*	*Cc08_g11010*	F: TGCTGAGGTTGGAGGGAAAA R: CGAACAAACAAGGGCAACAA
	CcABI2	*Cc06_g11740*	F: TACGGCTGTGGTTGGCATTA R: CTGCCCTTGAATCACCACAA
	CcAHG2	*Cc08_g16010*	F: AGAGCCCTGCTCCTGGTGAT R: GGTCATGCTACCGCGATCTT
	CcAHG3	*Cc02_g07430*	F: ACCGGAGGTGACGATAATCG R: CCCACAAGCTGTGTCATTGG
	CcHAB	*Cc04_g01620*	F: TGGCTTGTGGGATGTCATGA R: CGTTCTTCTTGTGCCAAAGCA
	CcHAI	*Cc01_g13400*	F: CATCGACGCTGCTTGTCAAT R: CCACCGCGTCTTCCATATCT
SnRK2	*CcSnRK2.1*	*Cc00_g19320*	F: TAGCCCCCGAGGTTCTCTCT R: TCACTCCGCAAGACCACACA
	CcSnRK2.2	*Cc07_g05710*	F: CGAGGATGAGGCTCGTTTTT R: GCTGGGCTTCCGTCTAACAA
	CcSnRK2.6	*Cc02_g18420*	F: GCATATATTGCGCCCGAAGT R: AAAGGGTATGCCCCCACAAG
	CcSnRK2.7	*Cc07_g14700*	F: AAGCCCAGAACCACGTCTCA R: GATTTGGGTTGGGAATGCAA
	CcSnRK2.8	*Cc10_g06790*	F1: AACATGTGCAGCGGGAGATT R1: CTCCTGCCGCATACTCCATT F2: CCGCTTCAAAGAGGTCTTGCT R2: TTCTCCTCCTGCCGCATACT
	CcSnRK2.10	*Cc02_g22790*	F: TCGATTCAAGGAGGTGGTGTT R: TTCCCCTCCAGCTGCATACT
	CcSnRK2.11	*Cc08_g11200*	F: AGGAACCTGACCCTCACCAA R: CCTGGGATTTTTGCCTCTTG
	CcSnRK2.12	*Cc00_g35430*	F: ACTTGAAGTTGGAAAACACATTTTTG R: GTCAAGGAAGGAATATGATGGGAAG
	CcSnRK2.13	Cc00_g07830	F: GGTGTTAGTTACTGTCATTCAATGGAA R: ACTTGAAGCTGGAAAACACACTTTT
	CcUBQ10	*Cc05_g13290*	F: AAGACAGCTTCAACAGAGTACAGCAT R: GGCAGGACCTTGGCTGACTATA

Table S1 Comparison of CcPYL protein sequences with orthologous sequences from *A. thaliana* (At), *C. sinensis* (Cs), *O. sativa* (Os), *S. lycopersicum* (Sl), *S. tuberosum* (St) and *V. vinifera* (Vv) plant species (Sp). NCBI accession numbers (ID), I (introns), aa (amino acid length), Ident. (Identity), Align. (Match/Aligned), QC (Query Cover), e-value and function information were obtained through BLASTp results. Coffee IDs were identified in Coffee Genome Hub (http://www.coffee-genome.org/) and Gene IDs in Phytozome 10.3 (http://phytozome.jgi.doe.gov/pz/portal.html) (…continue…).

ID	NCBI ID	*Gene ID*	Sp	I	aa	Ident.	Align.	QC	e-value	Function
Cc00_g17440	AAD25950.1	*AT2G40330.1*	*At*	0	175	47%	69%	91%	2,21E-47	hypothetical protein
	KDO68852.1	*orange1.1g038201m*	*Cs*	0	201	49%	69%	97%	2,53E-49	hypothetical protein CISIN
	EEE62745.1	*LOC_Os05g12260.1*	*Os*	0	196	48%	69%	84%	9,3E-42	hypothetical protein OsJ_17548
	XP_004249065.1	*Solyc10g076410.1.1*	*Sl*	0	203	52%	71%	92%	4,02E-50	PREDICTED: abscisic acid receptor PYL4-like
	XP_006359557.1	*PGSC0003DMG400029194*	*St*	0	214	52%	72%	92%	7,21E-51	PREDICTED: abscisic acid receptor PYL4-like
	XP_002264158.1	GSVIVG01032747001	*Vv*	0	227	54%	72%	95%	7,95E-54	PREDICTED: abscisic acid receptor PYL4
Cc00_g23730	AAD25950.1	*AT2G40330.1*	*At*	0	175	46%	68%	93%	6,88E-48	hypothetical protein
	KDO68852.1	*orange1.1g038201m*	*Cs*	0	201	48%	68%	97%	3,42E-48	hypothetical protein CISIN
	EEE62745.1	*LOC_Os05g12260.1*	*Os*	0	196	47%	68%	84%	1,57E-39	hypothetical protein OsJ_17548
	XP_004249671.1	*Solyc10g085310.1.1*	*Sl*	0	213	50%	71%	93%	3,9E-49	PREDICTED: abscisic acid receptor PYL4
	XP_006359557.1	*PGSC0003DMG400029194*	*St*	0	214	51%	71%	92%	5,34E-50	PREDICTED: abscisic acid receptor PYL4-like
	XP_002264158.1	GSVIVG01032747001	*Vv*	0	227	53%	71%	94%	3,01E-52	PREDICTED: abscisic acid receptor PYL4
Cc02_g01800	BAF00266.1	*AT5G53160.2*	*At*	2	188	82%	92%	90%	5E-100	hypothetical protein
	XP_006476396.1	orange1.1g028067m	*Cs*	2	197	78%	90%	98%	1,2E-103	PREDICTED: abscisic acid receptor PYL8-like
	NP_001046464.1	*LOC_Os02g15640.1*	*Os*	2	204	79%	92%	94%	2,71E-98	Os02g0255500
	XP_004234175.1	*Solyc03g007310.2.1*	*Sl*	2	185	83%	94%	98%	8,7E-111	PREDICTED: abscisic acid receptor PYL8
	NP_001275025.1	*PGSC0003DMG400009108*	*St*	2	185	82%	94%	98%	1,1E-109	abscisic acid receptor PYL8-like
	XP_002270037.3	*GSVIVG01028704001*	*Vv*	2	185	85%	94%	96%	2,1E-109	PREDICTED: abscisic acid receptor PYL8
Cc02_g05990	NP_565928.1	*AT2G40330.1*	*At*	0	215	57%	71%	91%	9,06E-74	abscisic acid receptor PYL6
	KDO68852.1	*orange1.1g038201m*	*Cs*	0	201	77%	87%	72%	5,75E-86	hypothetical protein CISIN
	NP_001055819.1	*LOC_Os05g39580.1*	*Os*	0	216	65%	75%	71%	4,62E-62	Os05g0473000
	XP_004235232.1	*Solyc03g095780.1.1*	*Sl*	0	201	75%	82%	88%	1,4E-99	PREDICTED: abscisic acid receptor PYL4-like
	XP_006353422.1	*PGSC0003DMG400023949*	*St*	0	218	74%	81%	91%	1,3E-104	PREDICTED: abscisic acid receptor PYL4-like
	XP_002264158.1	GSVIVG01032747001	*Vv*	0	227	68%	77%	90%	2,59E-91	PREDICTED: abscisic acid receptor PYL4
Cc02_g15060	AAD25950.1	*AT2G40330.1*	*At*	0	175	56%	79%	87%	5,89E-54	hypothetical protein
	KDO68852.1	*orange1.1g038201m*	*Cs*	0	201	60%	75%	93%	1,17E-60	hypothetical protein CISIN
	NP_001049838.1	*LOC_Os03g18600.1*	*Os*	0	229	55%	77%	93%	5,45E-52	Os03g0297600
	XP_004249671.1	*Solyc10g085310.1.1*	*Sl*	0	213	53%	79%	94%	3,56E-61	PREDICTED: abscisic acid receptor PYL4
	XP_006359557.1	*PGSC0003DMG400029194*	*St*	0	214	57%	79%	93%	1,09E-61	PREDICTED: abscisic acid receptor PYL4-like
	CAN72620.1	GSVIVG01013161001	*Vv*	0	172	62%	76%	97%	4,64E-70	hypothetical protein VITISV_004947

Table S1Continue… for legend see the previous page.

ID	NCBI ID	*Gene ID*	Sp	I	aa	Ident.	Align.	QC	e-value	Function
Cc02_g39180	3OQU	*AT1G01360.1*	*At*	2	205	75%	87%	96%	5,82E-87	Abscisic Acid Receptor Pyl9
	XP_006476396.1	*orange1.1g029200m*	*Cs*	2	197	75%	88%	96%	8,42E-90	PREDICTED: abscisic acid receptor PYL8-like
	NP_001054923.1	*LOC_Os05g12260.1*	*Os*	2	209	76%	88%	90%	5,84E-84	Os05g0213500
	XP_004231210.1	*Solyc01g095700.2.1*	*Sl*	2	186	83%	95%	91%	4,15E-98	PREDICTED: abscisic acid receptor PYL8
	NP_001284557.1	*PGSC0003DMG400000215*	*St*	2	186	81%	95%	91%	5,95E-96	abscisic acid receptor PYL8-like
	XP_010659134.1	*GSVIVG01019517001*	*Vv*	2	189	76%	89%	96%	6,96E-90	PREDICTED: abscisic acid receptor PYL8-like
Cc08_g02750	NP_193521.1	*AT4G17870.1*	*At*	0	191	68%	82%	83%	2,37E-87	abscisic acid receptor PYR1
	XP_006491739	*orange1.1g046151m*	*Cs*	0	187	55%	72%	78%	4,97E-66	PREDICTED: abscisic acid receptor PYL1-like
	NP_001065470.1	*LOC_Os10g42280.1*	*Os*	0	212	62%	73%	75%	2,29E-64	Os10g0573400
	XP_004245893.1	*Solyc08g076960.1.1*	*Sl*	0	231	72%	80%	97%	3,7E-110	PREDICTED: abscisic acid receptor PYR1-like
	NP_001284559.1	*PGSC0003DMG400017514*	*St*	0	231	70%	80%	97%	1,3E-107	abscisic acid receptor PYL1-like
	XP_002280361.1	*GSVIVG01013161001*	*Vv*	1	214	76%	85%	78%	7,3E-99	PREDICTED: abscisic acid receptor PYR1-like
Cc08_g10450	NP_180174.1	*AT2G26040.1*	*At*	0	190	72%	82%	95%	7,97E-85	abscisic acid receptor PYL2
	KDO80051.1	*orange1.1g046697m*	*Cs*	0	187	82%	88%	99%	1,4E-106	hypothetical protein CISIN
	NP_001172865.1	*LOC_Os02g13330.1*	*Os*	0	207	61%	74%	90%	2,27E-60	Os02g0226801
	XP_004253195.1	*Solyc12g095970.1.1*	*Sl*	1	190	80%	88%	99%	1,1E-103	PREDICTED: abscisic acid receptor PYL2-like
	XP_006360983.1	*PGSC0003DMG400029952*	*St*	1	188	84%	89%	98%	9,3E-107	PREDICTED: abscisic acid receptor PYL2-like
	XP_010648333.1	*GSVIVG01035362001*	*Vv*	0	185	82%	88%	95%	2,8E-99	PREDICTED: abscisic acid receptor PYL2
Cc08_g15960	NP_200128.1	*AT5G53160.2*	*At*	2	188	80%	91%	96%	2,32E-95	regulatory component of ABA receptor 3
	XP_006476396.1	*orange1.1g029200m*	*Cs*	2	197	87%	95%	97%	2,9E-106	PREDICTED: abscisic acid receptor PYL8-like
	NP_001046464.1	*LOC_Os02g15640.1*	*Os*	2	204	80%	90%	97%	1,92E-95	Os02g0255500
	XP_004245523.1	*Solyc08g082180.2.1*	*Sl*	2	189	85%	95%	97%	3E-105	PREDICTED: abscisic acid receptor PYL9
	XP_006343869.1	*PGSC0003DMG400012155*	*St*	2	189	85%	95%	97%	3E-105	PREDICTED: abscisic acid receptor PYL9-like
	XP_010659134.1	*GSVIVG01019517001*	*Vv*	3	189	90%	95%	97%	7,7E-111	PREDICTED: abscisic acid receptor PYL8-like

Table S2 Comparison of CcPP2C protein sequences with orthologous sequences from *A. thaliana* (At), *C. sinensis* (Cs), *O. sativa* (Os), *S. lycopersicum* (Sl), *S. tuberosum* (St) and *V. vinifera* (Vv) plant species (Sp). NCBI accession numbers (ID), I (introns), aa (amino acid length), Ident. (Identity), Align. (Match/Aligned), QC (Query Cover), e-value and function information were obtained through BLASTp results. Coffee IDs were identified in Coffee Genome Hub (http://www.coffee-genome.org/) and Gene IDs in Phytozome 10.3 (http://phytozome.jgi.doe.gov/pz/portal.html).

ID	NCBI ID	Gene ID	Sp	I	Len.aa	Ident.	Align.	Query cov.	e-value	Function inferred by BLAST
Cc01_g13400	NP_180499.1	*AT2G29380.1*	*At*	2	362	71%	83%	69%	2.3E-139	highly ABA-induced PP2C protein 3
	XP_006488392.1	*orange1.1g036852m*	*Cs*	2	429	64%	75%	98%	1.7E-159	PREDICTED: probable protein phosphatase 2C 78-like
	NP_001044788.1	*LOC_Os01g62760.1*	*Os*	3	414	55%	68%	70%	8.7E-108	Os01g0846300
	XP_004241211.1	*Solyc06g076400.2.1*	*Sl*	3	410	67%	76%	98%	7.2E-171	PREDICTED: probable protein phosphatase 2C 24
	XP_006350789.1	*PGSC0003DMG400030332*	*St*	3	410	67%	78%	97%	3E-173	PREDICTED: protein phosphatase 2C 37-like
	XP_002282608.1	*GSVIVT01024875001*	*Vv*	3	408	65%	74%	98%	2.3E-158	PREDICTED: probable protein phosphatase 2C 24
Cc02_g07430	NP_172223.1	*AT1G07430.1*	*At*	2	442	63%	77%	69%	1.3E-134	protein phosphatase 2C 3
	XP_006488392.1	*orange1.1g036852m*	*Cs*	2	429	59%	72%	99%	1.3E-150	PREDICTED: probable protein phosphatase 2C 78-like
	NP_001044788.1	*LOC_Os01g62760.1*	*Os*	3	414	58%	71%	73%	1.6E-121	Os01g0846300
	XP_004239911.1	*Solyc05g052980.2.1*	*Sl*	3	409	68%	78%	100%	0	PREDICTED: protein phosphatase 2C 37
	XP_006355694.1	*PGSC0003DMG400027196*	*St*	3	418	67%	77%	100%	0	PREDICTED: protein phosphatase 2C 37-like
	XP_002282703.1	*GSVIVT01016485001*	*Vv*	3	400	63%	73%	99%	7.9E-163	PREDICTED: protein phosphatase 2C 37-like
Cc04_g01620	NP_177421.1	*AT1G72770.1*	*At*	4	511	56%	74%	98%	0	protein phosphatase 2C 16
	XP_006465975.1	*orange1.1g009094m*	*Cs*	4	544	61%	74%	98%	0	PREDICTED: protein phosphatase 2C 16-like
	EEE54872.1	*LOC_Os01g40094.1*	*Os*	3	352	66%	77%	64%	4.8E-161	hypothetical protein OsJ_02363
	BAI39595.1	*Solyc03g121880.2.1*	*Sl*	4	544	73%	82%	98%	0	protein phosphatase 2C ABI2 homolog
	XP_006342955.1	*PGSC0003DMG400002573*	*St*	4	545	73%	83%	98%	0	PREDICTED: protein phosphatase 2C 16-like
	XP_002278167.2	*GSVIVT01016816001*	*Vv*	3	548	62%	74%	98%	0	PREDICTED: protein phosphatase 2C 16
Cc06_g11740	NP_177421.1	*AT1G72770.1*	*At*	4	511	63%	74%	61%	1.1E-142	protein phosphatase 2C 16
	KDO73536.1	*orange1.1g008890m*	*Cs*	4	550	49%	65%	100%	4.8E-161	hypothetical protein CISIN_1g008880mg
	NP_001046464.1	*LOC_Os01g40094.1*	*Os*	3	396	63%	75%	61%	6.9E-137	Os01g0583100, partial
	XP_004243737.1	*Solyc07g040990.2.1*	*Sl*	3	543	52%	68%	99%	0	PREDICTED: probable protein phosphatase 2C 50
	XP_006342333.1	*PGSC0003DMG400018004*	*St*	4	543	52%	68%	99%	0	PREDICTED: probable protein phosphatase 2C 6-like
	XP_002279140.1	*GSVIVT01015156001*	*Vv*	3	550	50%	66%	100%	5E-171	PREDICTED: protein phosphatase 2C 77
Cc08_g11010	NP_177421.1	*AT1G72770.1*	*At*	4	511	49%	63%	100%	2.6E-158	protein phosphatase 2C 16
	KDO73536.1	*orange1.1g008890m*	*Cs*	4	550	65%	78%	99%	0	hypothetical protein CISIN_1g008880mg
	NP_001065470.1	*LOC_Os01g40094.1*	*Os*	3	396	69%	80%	59%	2E-155	Os01g0583100, partial
	XP_004253091.1	Solyc12g096020.1	*Sl*	3	540	66%	79%	100%	0	PREDICTED: probable protein phosphatase 2C 53
	XP_006342498.1	*PGSC0003DMG400029297*	*St*	3	536	67%	79%	100%	0	PREDICTED: probable protein phosphatase 2C 6-like
	XP_010648365.1	*GSVIVT01035420001*	*Vv*	4	551	66%	78%	100%	0	PREDICTED: probable protein phosphatase 2C 53
Cc08_g16010	NP_172223.1	*AT1G07430.1*	*At*	2	442	47%	62%	64%	1.1E-80	protein phosphatase 2C 3
	KDO76517.1	*orange1.1g023178m*	*Cs*	4	286	57%	71%	65%	2.59E-95	hypothetical protein CISIN_1g023178mg
	NP_001043754.1	*LOC_Os01g46760.1*	*Os*	2	403	55%	70%	64%	2.77E-98	Os01g0656200
	XP_004240955.1	*Solyc06g051940.2.1*	*Sl*	2	442	57%	73%	67%	4.6E-103	PREDICTED: probable protein phosphatase 2C 51
	XP_006350568.1	*PGSC0003DMG400009112*	*St*	2	399	54%	70%	64%	1.88E-94	PREDICTED: probable protein phosphatase 2C 51-like
	XP_002266149.1	*GSVIVT01019525001*	*Vv*	2	393	57%	75%	70%	2E-107	PREDICTED: probable protein phosphatase 2C 51

Table S3 Comparison of CcSnRK2 protein sequences with orthologous sequences from *A. thaliana* (At), *C. sinensis* (Cs), *O. sativa* (Os), *S. lycopersicum* (Sl), *S. tuberosum* (St) and *V. vinifera* (Vv) plant species (Sp). NCBI accession numbers (ID), I (introns), aa (amino acid length), Ident. (Identity), Align. (Match/Aligned), QC (Query Cover), e-value and function information were obtained through BLASTp results. Coffee IDs were identified in Coffee Genome Hub (http://www.coffee-genome.org/) and Gene IDs in Phytozome 10.3 (http://phytozome.jgi.doe.gov/pz/portal.html).

ID	NCBI ID	*Gene ID*	Sp	I	Len.aa	Ident.	Align.	Query cov.	e-value	Function inferred by BLAST
Cc00_g07830	NP_172563.1	*AT1G10940.1*	*At*	9	363	80%	88%	70%	4.32E-76	serine/threonine-protein kinase SRK2A
	XP_006477070.1	*orange1.1g019433m*	*Cs*	8	341	84%	90%	70%	2.21E-77	PREDICTED: serine/threonine-protein kinase SAPK3-like
	NP_001050274.1	*LOC_Os03g27280.1*	*Os*	8	342	82%	88%	70%	2.78E-75	Os03g0390200
	XP_004245833.1	*Solyc08g077780.2.1*	*Sl*	8	339	82%	90%	70%	9.46E-77	PREDICTED: serine/threonine-protein kinase SAPK3
	XP_006359207.1	*PGSC0003DMG400026211*	*St*	8	339	82%	90%	70%	9.46E-77	PREDICTED: serine/threonine-protein kinase SAPK3-like
	XP_002262726.1	GSVIVT01004839001	*Vv*	8	340	83%	88%	70%	5.14E-76	PREDICTED: serine/threonine-protein kinase SAPK3
Cc00_g19320	NP_196476.1	*AT5G08590.1*	*At*	8	353	85%	93%	90%	0	serine/threonine-protein kinase SRK2G
	XP_006466196.1	orange1.1g018734m	*Cs*	8	354	84%	92%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2A-like
	NP_001052827.1	*LOC_Os04g35240.1*	*Os*	8	359	76%	85%	99%	0	Os04g0432000
	XP_004239628.1	*Solyc05g056550.2.1*	*Sl*	6	356	85%	91%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK7-like
	NP_001274892.1	*PGSC0003DMG400023803*	*St*	9	360	82%	90%	98%	0	serine/threonine-protein kinase SRK2B-like
	XP_002267922.1	*GSVIVT01022427001*	*Vv*	8	355	85%	90%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2A
Cc00_g35430	NP_172563.1	*AT1G10940.1*	*At*	9	363	87%	93%	94%	8.76E-71	serine/threonine-protein kinase SRK2A
	KDO57025.1	*orange1.1g024336m*	*Cs*	8	269	92%	95%	94%	7.14E-75	hypothetical protein CISIN
	BAT12097.1	*LOC_Os10g41490.1*	*Os*	8	289	88%	94%	94%	1.8E-72	Os10g0564500
	XP_004245833.1	*Solyc08g077780.2.1*	*Sl*	8	339	92%	95%	94%	5.72E-74	PREDICTED: serine/threonine-protein kinase SAPK3
	XP_006359207.1	*PGSC0003DMG400026211*	*St*	8	339	92%	95%	94%	7.26E-74	PREDICTED: serine/threonine-protein kinase SAPK3-like
	XP_002262726.1	*GSVIVT01004839001*	*Vv*	8	340	92%	94%	94%	3.02E-73	PREDICTED: serine/threonine-protein kinase SAPK3
Cc02_g18420	NP_567945.1	*AT4G33950.1*	*At*	9	362	87%	93%	99%	0	calcium-independent ABA-activated protein kinase
	KDO49166.1	*orange1.1g017933m*	*Cs*	7	363	91%	97%	99%	0	hypothetical protein CISIN
	NP_001050653.1	*LOC_Os03g41460.1*	*Os*	6	362	87%	92%	99%	0	Os03g0610900
	XP_004230794.1	*Solyc01g108280.2.1*	*Sl*	9	362	93%	98%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2E
	NP_001275318.1	*PGSC0003DMG400025895*	*St*	10	362	93%	97%	99%	0	serine/threonine-protein kinase SRK2E-like
	XP_002284959.1	*GSVIVT01031806001*	*Vv*	8	363	90%	96%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK10

Table S3 Continue…for legend see the previous page.

ID	NCBI ID	*Gene ID*	Sp	I	Len.aa	Ident.	Align.	Query cov.	e-value	Function inferred by BLAST
	AAM67112.1	*AT1G60940.1*	*At*	8	361	84%	91%	99%	0	putative serine/threonine-protein kinase
	XP_006471015.1	*orange1.1g018734m*	*Cs*	8	351	87%	92%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2A-like
	NP_001052827.1	*LOC_Os04g35240.1*	*Os*	8	359	83%	93%	99%	0	Os04g0432000
	XP_004230475.1	*Solyc01g103940.2.1*	*Sl*	9	361	88%	94%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2B
	NP_001274892.1	*PGSC0003DMG400023803*	*St*	9	360	91%	96%	99%	0	serine/threonine-protein kinase SRK2B-like
Cc02_g22790	XP_002269221.1	*GSVIVT01023339001*	*Vv*	8	356	91%	95%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2A
	NP_201489.1	*AT5G66880.1*	*At*	8	361	83%	91%	99%	0	serine/threonine-protein kinase SRK2I
	KDO49166.1	*orange1.1g017933m*	*Cs*	7	363	84%	93%	99%	0	hypothetical protein CISIN
	NP_001050653.1	*LOC_Os03g41460.1*	*Os*	6	362	81%	91%	99%	0	Os03g0610900
	XP_004232055.1	*Solyc02g090390.2.1*	*Sl*	8	352	86%	95%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2I
	XP_006338224.1	*PGSC0003DMG400025895*	*St*	8	352	86%	95%	99%	0	PREDICTED: serine/threonine-protein kinase SRK2I-like
Cc07_g05710	XP_002284959.1	*GSVIVT01031806001*	*Vv*	8	363	82%	91%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK10
	NP_567945.1	*AT4G33950.1*	*At*	9	362	73%	87%	93%	1.2E-173	calcium-independent ABA-activated protein kinase
	XP_006466260.1	orange1.1g019628m	*Cs*	8	341	82%	90%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK2-like
	NP_001060312.1	*LOC_Os07g42940.1*	*Os*	8	339	79%	88%	99%	0	Os07g0622000
	XP_010312635.1	Solyc04g012160.2.1	*Sl*	8	345	80%	87%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK2
	NP_001274912.1	*PGSC0003DMG400023636*	*St*	8	344	79%	87%	99%	0	serine/threonine-protein kinase SAPK2-like
Cc07_g14700	XP_003632469.1	*GSVIVT01003419001*	*Vv*	8	338	83%	91%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK2
	NP_567945.1	*AT4G33950.1*	*At*	9	362	72%	87%	95%	9.9E-180	calcium-independent ABA-activated protein kinase
	XP_006477070.1	*orange1.1g019433m*	*Cs*	8	341	84%	91%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK3-like
	BAD17999.1	*LOC_Os10g41490.1*	*Os*	9	334	85%	93%	90%	0	serine/threonine protein kinase SAPK3
	XP_004245833.1	*Solyc08g077780.2.1*	*Sl*	8	339	83%	91%	100%	0	PREDICTED: serine/threonine-protein kinase SAPK3
	XP_006359207.1	*PGSC0003DMG400026211*	*St*	8	339	82%	91%	100%	0	PREDICTED: serine/threonine-protein kinase SAPK3-like
Cc08_g11200	XP_002262726.1	*GSVIVT01004839001*	*Vv*	8	340	84%	90%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK3
	NP_974170.1	*AT1G78290.3*	*At*	5	343	83%	91%	88%	0	serine/threonine-protein kinase SRK2C
	KDO81023.1	*orange1.1g019628m*	*Cs*	8	338	83%	90%	99%	0	hypothetical protein CISIN
	NP_001050274.1	*LOC_Os03g27280.1*	*Os*	8	342	77%	87%	99%	0	Os03g0390200
	XP_004237936.1	*Solyc04g074500.2.1*	*Sl*	8	336	80%	90%	98%	0	PREDICTED: serine/threonine-protein kinase SAPK2
	NP_001275016.1	*PGSC0003DMG400030830*	*St*	8	335	80%	90%	98%	0	serine/threonine-protein kinase SAPK2-like
Cc10_g06790	XP_003634478.1	*GSVIVT01009074001*	*Vv*	8	335	81%	90%	99%	0	PREDICTED: serine/threonine-protein kinase SAPK2

CHAPTER 2

Gene expression profiles in *Coffea arabica* and *Coffea canephora* leaves revealed transcriptional regulations of key genes involved in ABA signaling

ABSTRACT

In response to exogenous ABA, 17 genes were expressed in leaves of *Cc* and *Ca* plants. Several genes were differentially expressed in the D^T clone 14 either in control condition or after 24h with ABA treatment. Under control condition, five genes were higher expressed as in the *Cc* as in *Ca* D^T plants. The kinase *CcSnRK2.6* was highlighted as gene specifically expressed in the *Cc* plants (D^T and D^S) after 72h of ABA treatment. Overall, it was observed that ABA signaling pathway is delayed in the D^S *C. arabica* Rubi. Those molecular evidences corroborated with microscopies analyses which showed that the D^T clone 14 was more efficient to control the stomatal closure than other coffee plants in response to ABA treatment. All these evidences will help us to identify the genetic determinism of drought tolerance through ABA pathway essential to obtain molecular markers that could be used in coffee breeding programs.

Keywords: ABA. *Coffea arabica*. *Coffea canephora*. Guard cells

GENE EXPRESSION PROFILES IN *COFFEA ARABICA* AND *COFFEA CANEPHORA* LEAVES REVEALED TRANSCRIPTIONAL REGULATIONS OF KEY GENES INVOLVED IN ABA SIGNALING.

INTRODUCTION

Stomatal guard cells are functionally specialized epidermal cells usually located on plant aerial organs which control gas exchanges between plant and the surrounding atmosphere. These guard cells have developed mechanisms to sense and respond to various endogenous and environmental stimuli (Hetherington; Woodward, 2003; Gray, 2005; Masle et al., 2005).

The role of ABA in guard cell regulation after drought response has been extensively studied since a long time (Schroeder et al., 2001a; Nilson; Assmann, 2007; Sirichandra et al., 2009). For example, applications of exogenous ABA was show to stimulate stomatal closure in the wilty tomato *flacca* mutant deficient in ABA (Imber; Tal, 1970; Tal et al., 1970), as well as in *Xanthium* (Jones; Mansfield, 1970). The opening and closing of the stomatal pore are regulated by osmotic pressure of guard cells envolving dynamic changes in the intracellular concentrations of inorganic ions and sugars (Sirichandra et al., 2009).

It is well known that the ABA PYR/PYL/RCAR receptors play a key role for the whole-plant stomatal adjustments and responses to low humidity, darkness, and elevated CO_2, for example (Merilo et al., 2013). Under drought, some plant species maintain leaf water potential (isohydric behavior) while other favor stomatal conductance to maintain CO_2 assimilation (anisohydric behaviour). The first mechanism results of the enhancement of the ABA effect on stomatal conductance (g_s) by low Ψ_{leaf} (Tardieu; Simonneau, 1998). ABA production induced by low Ψ_{leaf} is thought to prevent stomata to reach their maximal opening by a transduction network involving ABI1 and ABI2 protein phosphatases 2C and

the OST2 and SLAC1 effectors (Kim et al., 2010). On the other hand, vascular ABA decreases K_{leaf} putatively by inactivating aquaporins such as the plasm membrane intrinsic proteins (PIPs) (Shatil-Cohen et al., 2011), through a transduction pathway distinct from the network already described. This conceptual model for the dual action of ABA on stomata closure has been recently proposed (Pantin et al., 2013).

Regarding the key roles of tripartite system in higher plants, the following scientific questions arisen concerning coffee:

- how the *PYL*/*PP2C*/*SnRK2* genes are expressed in leaves of coffee plants in response to exogenous ABA?
- are they differentially expressed in D^T and D^S clones?
- does it exist different expression profiles of these genes in *C. arabica* and *C. canephora*?
- is it possible to correlate the expression profiles of the genes with stomatal responses in the D^T and D^S clones of *C. canephora* and *C. arabica*?
- Is it possible to correlate the expression profiles of these genes with those observed under drought conditions for the *C. canephora* plants (chapter I)?

Aiming to get the answers to these questions, the main objectives of this work were:

(i) to cultivate in hydroponic conditions *C. arabica* and *C. canephora* plants;

(ii) to characterize the expression profile in time-course of genes belonging to the tripartite system (*PYR -PP2C-SnRK2*) in leaves of D^T and D^S clones of *C. canephora* and *C. arabica* submitted to exogenous ABA treatment;

(iii) to study the effects of exogenous ABA on stomatal aperture in *C. canephora* and *C. arabica* plants;

MATERIAL AND METHODS

Plant material

D^T (14, 73 and 120) and D^S (22) clones of *C. canephora* corresponded to those previously described in the chapter I were grown in greenhouse conditions (under controlled temperature 25°C, relative humidity of 70% and photosynthetic flux PPF 900 $\mu mol^{-2}s^{-1}$) in small containers at UFV (University of Viçosa-UFV, Minas Gerais, Brazil) and used for stem cuttings to generate *C. canephora* plantlets to be tested in hydroponic conditions. Plants of the D^T (IAPAR59) and D^S (Rubi) cultivars of *C. arabica* were obtained from seeds harvested in the experimental fields of Embrapa Cerrados that were germinated in deionized water.

Hydroponic condition for ABA experiment

For both D^T and D^S genotypes of *C. canephora* and *C. arabica*, 2 plants were used as biological repetitions. The plants were hydroponically grown in culture room with 150-200 µmol photon/m^2/s light intensity, 12/12 dark/light hours, 70% relative humidity at 24±1°C in pH 5.5 adjusted Hoagland solution (Hoagland; Arnon, 1950) ¼ strength. For hydroponic assay, *C. canephora* and *C. arabica* plants of 6 and 3 months-old, respectively, were transferred from the greenhouse to culture room in individual pots (300 mL) immersed with nutritive solution that was renewed weekly. ABA assays were performed one month after plants acclimation in hydroponic conditions by adding ABA to a final concentration of 500 µM in the nutritive Hoagland solution.

RNA extraction

RNAs were extracted as previously described (Marraccini et al., 2012) from the first pair of leaf of coffee plants grown in hydroponic conditions where

they were submitted to ABA treatment during 3 days. The samples were collected at 11:30 am in control (Hoagland ¼ strength w/o ABA) and under ABA (500 µM) conditions at the first and third days. All purified RNAs were quantified using a NanoDrop 1000 Spectrophotometer (Waltham, MA, USA). Contaminant genomic DNA was eliminated from purified RNAs by RQ1 RNase-free DNase (Promega) treatment according to the fabricant. RNA integrity was verified by agarose gel electrophoresis with ethidium bromide staining. Synthesis of the first-strand cDNA was done by treating 2.4µg of total RNA with the ImProm-II Reverse Transcription System and oligo (dT15) according to the manufacturer's recommendations (Promega).

Real time qPCR assays

Genomic DNA was eliminated from purified RNAs by RQ1 RNase-free DNase (Promega) treatment according to the fabricant. RNA integrity was verified by agarose gel electrophoresis with ethidium bromide staining. Synthesis of the first-strand cDNA was done by treating 2.4µg of total RNA with the ImProm-II Reverse Transcription System and oligo (dT15) according to the manufacturer's recommendations (Promega). Real-time qPCR assays were carried out with the synthesized single-stranded cDNA described above and using the protocol recommended for 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). cDNA preparations were diluted (1/20) and tested by qPCR using 48 primer pairs designed for the 24 candidate genes of the tripartite systems. Primer pairs were designed using the Primer Express software (Applied Biosystems) and preliminarily tested for their specificity with a cDNA mix from roots. The qPCR was performed with 1µl of diluted single-stranded cDNA and 0.2 µM (final concentration) of each primer in a final volume of 10µl with 1x SYBR green fluorochrome (SYBRGreenqPCR Mix-UDG/ROX, Invitrogen). The reaction mixture was incubated for 2 min at 50°C and 5 min at

95°C (UDG step), followed by 40 amplification cycles of 3 s at 95°C, 30 s at 60°C. Data were analyzed using the SDS 2.1 software (Applied Biosystems) to determine the cycle threshold (Ct) values. Specificity of the PCR products generated for each set of primers was verified by analyzing the Tm (dissociation) of amplified products. Gene expression levels were normalized to expression level of ubiquitin (*CcUBQ10*) as a constitutive reference (Barsalobres-Cavallari et al., 2009). Expression was expressed as relative quantification by applying the formula $(1+E)^{-\Delta\Delta Ct}$, where $\Delta Ct_{\text{target}} = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{internal calibrator}}$, the internal calibrator always being the 14I sample with relative quantification equal to 1. Data are presented as the mean ± standard error of the mean. Graphs are generated and analyzed using GraphPad Prism ©.

Microscopic analyses

For each genotype, the first pair of leaf from two different plants was used for transversal sections. Two different areas of the leaves were collected twice at mid-day at 11:30 am before ABA treatment (control) and at the same time in each one of the three days of assay. Additional sample was collected in the third day at 6 pm. Immediately after harvest, the material was fixed in FAA 50% (formaldehyde, acetic acid and ethanol) solution for both scanning electronic (SEM) or optical microscopy. After 24 hours of incubation, samples were dehydrated through a graded series of ethanol until 70% and then cleared in sodium hypochlorite 2,5% over 2 hours before to be analyzed by microscopy (Leica DM 750 microscope). For optical analyses, images were treated using the Leica Application Suite 3.0 LasEz software and stomatal densities were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). For calculation of stomatal aperture, the length of one hundred stomatal guard cells was measured in leaves of each genotype under control and ABA treatments.

RESULTS

Expression profiles of CcPYL-PP2C-SnRK2 genes in hydroponic-grown plants treated with exogenous ABA

The expression profiles of the tripartite system *CcPYL-PP2C-SnRK2* genes were analyzed in coffee plants growing under hydroponic condition and submitted to ABA treatment (Figure). For this purpose, plants of *C. canephora* and *C. arabica* were incubated during three days in nutritive solution containing 500 µM of ABA. Leaf samples were collected for all plants (*C. canephora*, D^T: clone 14 and D^S: clones 22; *C. arabica* D^T I59 and D^S Rubi) before assay (control, without ABA) and after one (24 hours) and three days (72 hours) under ABA treatment. These samples were used for qPCR (Figure 3 and Figure 4) experiments and microscopy analyses (Figure 5).

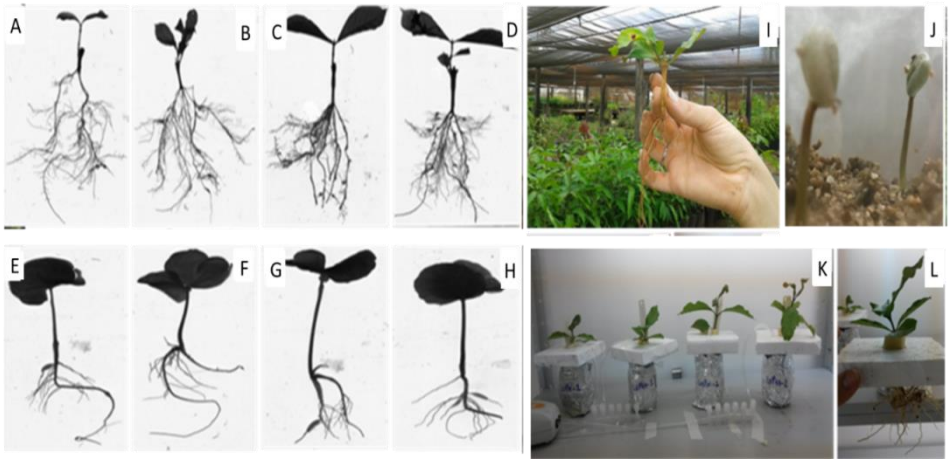


Figure 1 Experimental condition for hydroponic assays. *C. canephora* D^T clone 14 (A-B) and D^S clone 22 (C-D) were originated from stem cuttings (I). The plantlets of D^T cultivar IAPAR59 (E-F) and D^S cultivar Rubi (G-H) of *C. arabica* were originated from germinated seeds (J). Images of individual plantlets (A-H) were generated using the WinRhizo software prior ABA treatment. All plants were hydroponically grown (K-L) in controlled growth chamber.
Source From author (2016)

Expression of PYL genes

Among the nine *CcPYL* genes previously identified (chapter I), *PYL2*, *CcPYL7a, PYL7b* and *PYL13* were not expressed in leaves of *C. canephora* and *C. arabica* genotypes either under control or ABA treatments. However, the *PYR1*, *PYL4*, *PYL8a*, *PYL8b* and *PYL9* genes were expressed in low level in leaves of all coffee genotypes grown under hydroponic conditions without ABA (Figure 2).

In *C. canephora*, up-regulated expression of *CcPYR1* and *CcPYL8b* genes was clearly observed after 24 hours of ABA treatment specifically in leaves of the D^T clone 14 but not in those of D^S clone 22. For both clones, leaf expression of *CcPYR1*, *CcPYL4*, *CcPYL8a*, *CcPYL8b* and *CcPYL9* genes decrease hereafter to be undetectable at 72h of ABA treatment.

In *C. arabica*, *CaPYL8a* was the only gene showing up-regulated expression under at 24h of ABA treatment in I59. At 72h of ABA treatment, leaf expression of *CaPYR1*, *CaPYL4*, *CaPYL8a* and *CaPYL9* genes was no more detected in I59 but observed in Rubi. In both genotypes, expression of *CaPYL9* gene was undetected in control and at 24h of ABA treatment and considered as low at 72h (Figure 2).

Altogether, this study clearly highlighted the existence of different *PYL* expression profiles between D^T and D^S clones in each coffee species but also between *C. canephora* and *C. arabica* plantlets, mainly regarding the time-course of *PYL* expression upon ABA treatments.

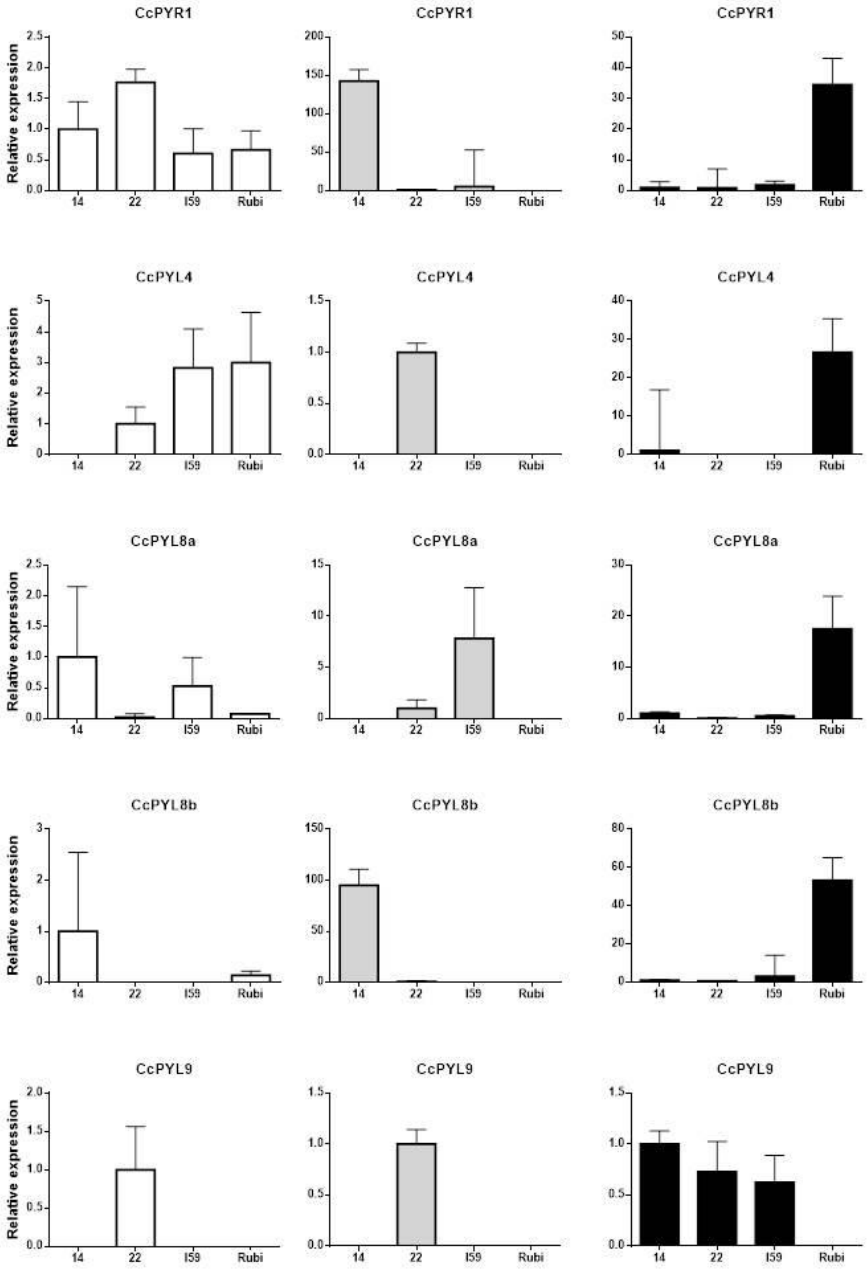


Figure 2 Expression profiles of *PYL* genes in leaves of *C. canephora* D^T (clone 14) and D^S (clone 22) and *C. arabica* D^T (I59) and D^S (Rubi) plants in response to exogenous ABA. (…continue…)

RNAs were extracted from leaves of coffee plantlets without exogenous ABA (control, white bars) as well as after 24 (grey bars) or 72 hours (black bars) under ABA treatment (500 µM), *PYL* genes studied corresponded to *PYR1*, *PYL4*, *PYL8a*, *PYL8b* and *PYL9* genes. Values are the mean of at least three technical repetitions ± SD which are standardized independently with *UBQ10* (ubiquitin) as reference gene. The clone 14 was choose as preferential internal calibrator (RE=1).

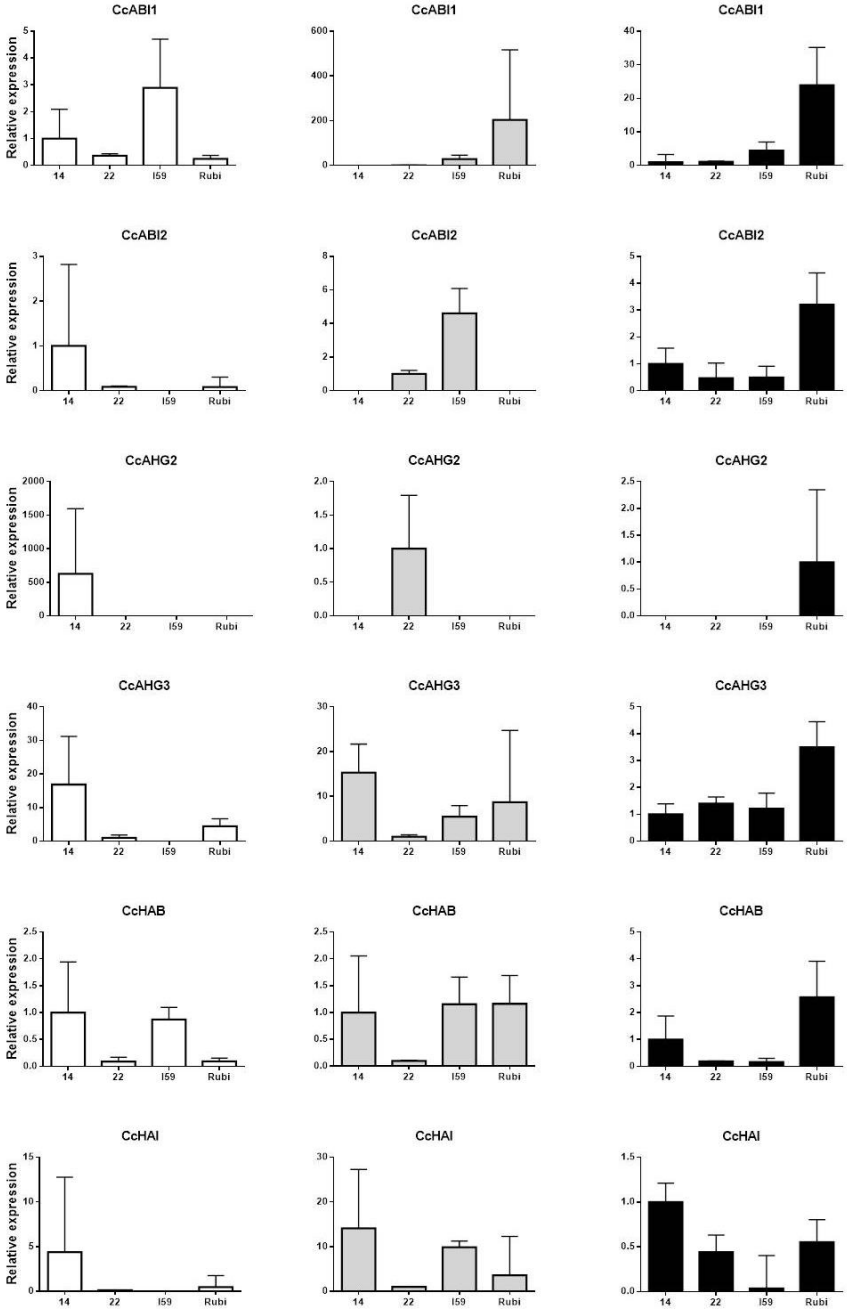


Figure 3 Expression profiles of *PP2C* genes in leaves of *C. canephora* D^T (clone 14) and D^S (clone 22) and *C. arabica* D^T (I59) and D^S (Rubi) plants in response to exogenous ABA. (…continue…)

RNAs were extracted from leaves of coffee plantlets without exogenous ABA (control, white bars) as well as after 24 (grey bars) or 72 hours (black bars) under ABA treatment (500 µM), *PP2C* genes studied corresponded to *ABI1-2, AGH2-3, HAB, HAI* genes. Values are the mean of at least three technical repetitions ± SD which are standardized independently with *CcUBQ10* (ubiquitin) as reference gene. The clone 14 was choose as preferential internal calibrator (RE=1).

Expression of PP2C genes

Among the six *CcPP2C* genes previously identified (chapter I), and except *AHG2 in C. arabica*, all (*CcABI1* and *2*, *CcAHG3*, *CcHAB* and *CcHAI*) were expressed in leaves of both *C. canephora* and *C. arabica* plantlets in hydroponic prior to ABA treatment (Figure 3).

In *C. canephora*, it is worth noting the higher expression level of *CcAHG2*, *CcAHG3* and *CcHAI* genes in D^T clone 14 compared to D^S clone 22 under unstressed conditions. After 24h of ABA treatment, *CcAHG2* leaf expression decreased significantly in D^T clone 14. However, expression profiles of all other genes were similar to those observed in the control condition, and continued to be low at 72h of ABA.

In *C. arabica* and whatever the genotype, expression levels of *PP2C* genes were considered as low under control condition. After 24h of ABA treatment, the main changes in expression profiles were observed for *ABI1* gene that was highly up-regulated in cultivar Rubi but not in I59. Even though, *ABI1* expression levels decreased hereafter, to be lower than those measured at 24h, *ABI1* expression continued to be higher in Rubi than in I59 at 72h of ABA treatment. Interestingly, *AHG2* expression was not detected in leaf of both cultivars under control condition and after 24h of ABA, but was detectable at 72h of ABA treatment only in leaves of Rubi D^S cultivar. For other *PP2Cs*, ABA treatments did not modify significantly gene expression profiles that were considered as low and relatively stable in both cultivars.

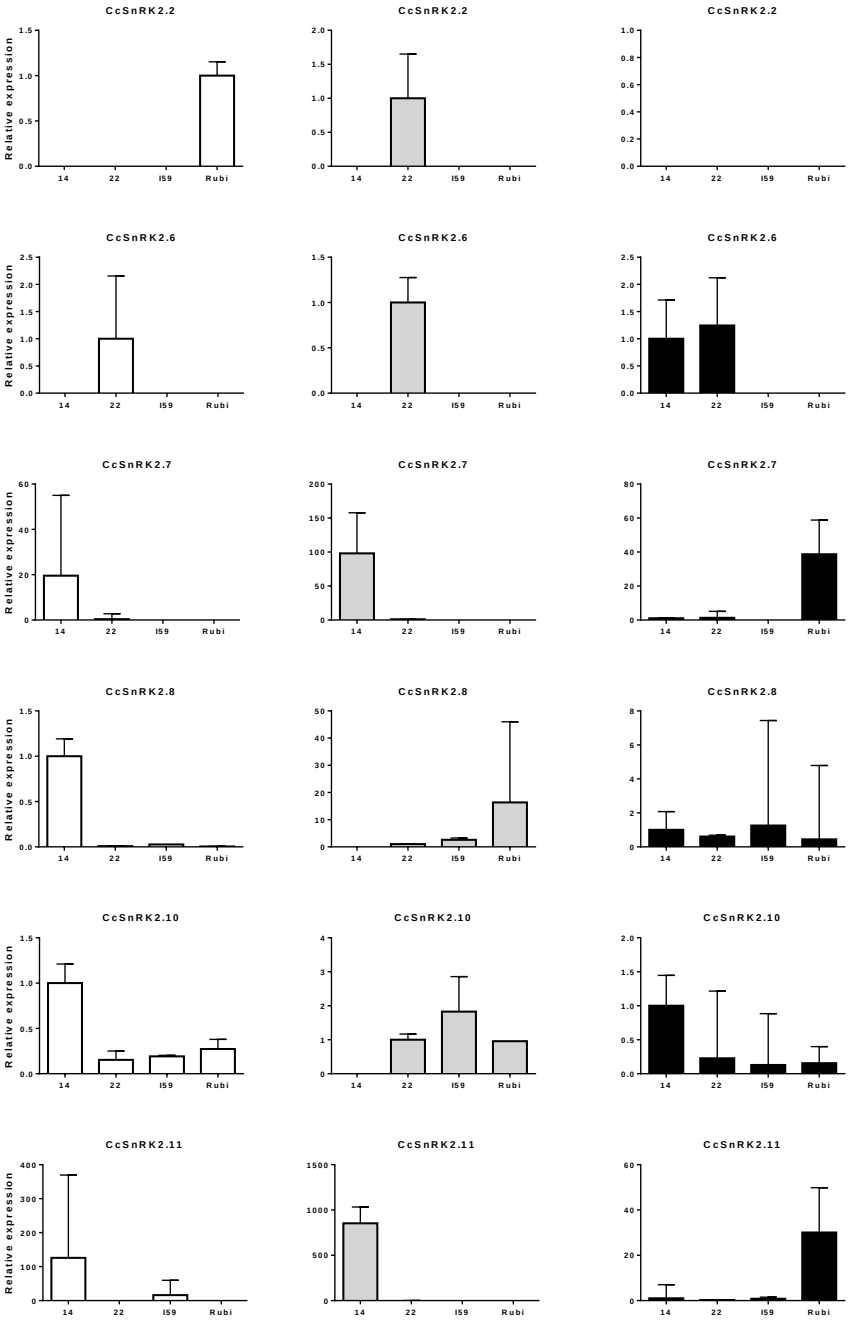


Figure 4 Expression profiles of *SnRK2* genes in leaves of *C. canephora* D^T (clone 14) and D^S (clone 22) and *C. arabica* D^T (I59) and D^S (Rubi) plants in response to exogenous ABA. (…continue…)

RNAs were extracted from leaves of coffee plantlets without exogenous ABA (control, white bars) as well as after 24 (grey bars) or 72 hours (black bars) under ABA treatment (500 µM), *SnRK2* genes studied corresponded to *SnRK2.2, SnRK2.6, SnRK2.7, SnRK2.8, SnRK2.10, SnRK2.11* genes. Values are the mean of at least three technical repetitions ± SD which are standardized independently with *CcUBQ10* (ubiquitin) as reference gene. The clone 14 was choose as preferential internal calibrator (RE=1).

Expression of SnRK2 genes

Among the *CcSnRK2* previously identified (chapter 1), six of them (*CcSnRK2.2*, *CcSnRK2.6*, *CcSnR2.7*, *CcSnRK2.8*, *CcSnRK2.10* and *SnRK2.11*) were studied by qPCR experiments (Figure 30). While *CcSnRK2.6* gene was expressed in both D^T and D^S clones of *C. canephora*, it is worth noting that expression of this gene was not detected in both cultivars of *C. arabica*. On the other hand, we can point out that *CcSnRK2.10* expression profiles detected in all coffee genotypes were not greatly affected by ABA treatments. For other *SnRK2* genes, the main differences observed between coffee species, genotypes and ABA treatments are given below.

In *C. canephora*, expression of *CcSnRK2.2* was undetectable in leaves of both clones under control condition. Under these conditions, it is worth noting higher expression level in D^T clone 14 than D^S clone 22 mainly for *CcSnR2.7* and *CcSnR2.11* genes, and to a lesser extend for *CcSnRK2.8*, and *CcSnRK2.10*. The contrary was observed for *CcSnRK2.6* that had higher expression in D^S clone 22 than in D^T clone 14. Expression of *CcSnRK2.7* and *CcSnRK2.11* genes appeared greatly up-regulated in D^T clone 14 after 24h of ABA treatment, and decreased drastically hereafter at 72h of ABA treatment. In parallel and whatever the tested conditions, expression of *CcSnRK2.7* and *CcSnR2.11* gene was always undetected in leaves of D^S clone 22.

In *C. arabica*, it is worth noting that expression profiles of all *SnRK2* genes were always low, up to undetectable in the D^S cultivar IAPAR59. In the D^S

cultivar Rubi, expression of *SnRK2.2* clearly decreased after 24h of ABA treatment while the contrary was observed for *SnRK2.8* gene. In this cultivar, expression of *SnRK2.7* and *SnRK2.11* was highly up-regulated after 72h of ABA treatment, while *SnRK2.8* gene expression decreased.

Effects of ABA treatments on stomatal closure in D^T and D^S clones of C. canephora.

In leaves, the D^T and D^S plants of *C. canephora* presented differences in stomatal cell responses under ABA treatment (Figure 5).

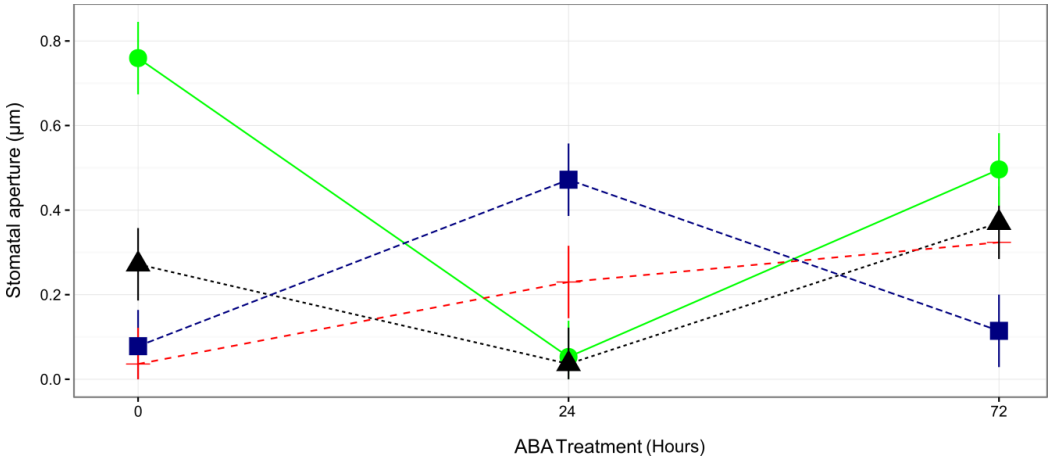


Figure 5 Evaluation of ABA effect in modulating the guard cells stomatal aperture in coffee leaves. The stomatal aperture length was measured in the guard cells of *C. canephora* D^T 14 (circles) and D^S 22 clones (triangles) and *C. arabica* D^T I59 (squares) and D^S Rubi (crosses) cultivars in 24 and 72 hours after application of exogenous ABA 500 µM solution and under control conditions (without ABA, 0h). The stomatal aperture values are given as an average of a hundred cells measurements for each clone/cultivar.

Under control condition, the *C. canephora* D^T clone 14 showed in average larger stomatal aperture than D^S clone 22 (Figure 5). After 24h and 72h of ABA treatment, no significant difference was observed between D^T and D^S clones. When analyzed separately, either clones showed significant responses to ABA

treatments. After the first 24h, the clones D^T 14 and D^S 22 showed an increase of stomatal closure (decrease in guard cells stomatal aperture). After 72h, this process was fully reversed in D^S clone 22, where none significant difference was observed in stomatal aperture between 0h and 72h, and partially reversed in D^T clone 14, where despite of stomatal aperture increase significant differences were still observed between 0h and 72h. In contrast, *C. arabica* cultivars D^T I59 and D^S Rubi presented smaller stomatal aperture values than *C. canephora* clones under control conditions. Significant changes in stomatal aperture were observed between guard cells of D^T I59 and D^S Rubi after 24h and 72h of ABA treatment. The D^T I59 cultivar increases guard cells opening following 24h of ABA exogenous stimulation and further increases guard cells closing during the last 48h. None statistically significant changes in stomatal aperture of D^S Rubi guard cells were promoted with exogenous ABA stimulation. Clearly, exogenous ABA stimulation affected distinctively the stomatal control in guard cells of D^T *C. arabica* and *C. canephora* plants. Overall, guard cells of *C. canephora* clones dispose of larger stomatal aperture in natural conditions and responds to ABA stimulation by inducing stomatal closure in the first 24h but followed by stomatal reopening in the last 48h. Distinctively, in the first 24h guard cells of *C. arabica* D^T I59 induces stomatal opening upon ABA stimulation and further promotes stomatal closure in the 48h.

DISCUSSION

In this part of the work, we focused our attention to study the effects of exogenous ABA to affect the expression of *PYL-PP2C-SnRK2* genes of ABA tripartite in D^T and D^S genotypes of *C. canephora* and *C. arabica*. Among the nine *PYL* genes characterized in *C. canephora* (Chapter I), *PYR1*, *PYL4*, *PYL8a* and *PYL8b*, *PYL9* were the genes that presented the most relevant differences of expression profiles between *C. canephora* and *C. arabica* species, but also

between D^T and D^S genotypes of the same coffee species and ABA treatments. Except for *PYL2*, the results presented here are in accordance with those described as expressed genes in leaves of *C. canephora* plants under I or NI conditions (Chapter I).

Regarding the first step of ABA tripartite system, we clearly highlighted that the D^S Rubi cultivar of *C. arabica* up-regulated the expression of *CcPYLs* genes latter (after 72 hours of ABA) compared to earlier responses observed for the same genes in other genotypes. Besides that, it is worth noting that *PYL9* gene expression was not detected in control or under ABA treatment only for *C. arabica* var. Rubi. It was recently suggested that *PYL9* promoted drought resistance not only by limiting transpiration water loss but also, by causing summer dormancy-like responses, such as senescence (Zhao et al., 2016). In plants, leaf senescence increases the transfer of nutrients to developing and storage tissues. Moreover, transgenic tobacco showed that delayed leaf senescence increases plant resistance to drought (Rivero et al., 2007). These evidences also corroborate with the physiological and molecular responses previously observed for the D^T and D^S *C. canephora* and *C. arabica* plants submitted to drought conditions (Pinheiro et al., 2005; Marraccini et al., 2011; Mofatto et al., 2016). The *C. canephora* D^S clone 22 maintained the same expression levels of *PYL9* in control or ABA treatments while the D^T genotypes of *C. canephora* or *C. arabica* up-regulated *PYL9* expression in control conditions and ABA treatments.

It is important highlighting that the *CcPYR1* and *CcPYL8b* genes are highly up-regulated mainly in the *C. canephora* D^T clone 14 in a fast response (24 h to exogenous ABA. Previous microarray data and GUS expression studies have shown that *PYR1* and *PYL8* were expressed in guard cells (Gonzalez-Guzman et al., 2012). We have previously shown (Chapter I) that *CcPYR1* was significantly down-regulated under drought in all clones of *C. canephora* except in D^T clone

14 that maintained similar expression levels in leaves under I or NI conditions. *CcPYL8b* expression levels also not presented significant difference between I or NI in clone 14. *Arabidopsis* transgenic *PYL8-OX* plants were generated and showed drought tolerance phenotype through enhanced stomatal closure in response to ABA (Lim et al., 2013). HAB1 interacts with PYL8 and also with PYR1, however, the interaction with PYL8 was not ABA-dependent while with PYR1 did not occur in the absence of exogenous ABA in Y2H interaction (Santiago, et al. 2009b; Park et al. 2009). Recent work showed that subcellular localization of PYL8 changes in response to ABA (Lee et al., 2015). PYL8 protein moves into the nucleus in response to ABA and the subcellular localization of PYL8 is regulated by abiotic stress signals. These result were also observed for PYL9 (Lee et al., 2015).

Interestingly, under control conditions *ABI1* and *HAB* genes had higher expression levels in D^T clone 14 and Rubi cultivar of *C. canephora* and *C. arabica*, respectively. With ABA treatment, the clone 14 maintained expression levels of *CcHAB* gene at 24 and 72 h of ABA treatment. In contrast, I59 maintained *HAB* expression level during the first 24 h of ABA treatment, since its expression decreased at 72 h in this genotype. In contrast, in leaves of the *C. arabica* D^S Rubi, the *HAB* gene was upregulated after 24 h ABA treatment and the expression levels continue to increase at 72 h. The D^S clone 22 showed a uniform low expression of this gene from control to 72 hours ABA treatment.

HAB1 was originally cloned on the basis of sequence homology to *ABI1* and *ABI2*. In the case of *ABI1/ABI2*, the level of expression in response to ABA is notably higher for *ABI1* than *ABI2* (Saez et al., 2003). This evidence was in accordance with our results where *CcABI1* was most expressed than *CcABI2* gene under ABA treatment *for C. arabica* plants. After 72 hours, the most expressed gene in Rubi was *CcABI1*. However, there was a peak of expression in this gene in Rubi after 24 hours under ABA treatment which suggests that this PP2C was

highly expressed in this clone which could repress the transcription of kinases as *SnRK2.2* and *SnRK2.6*. In this sense, the drought-response genes could be later activated in ABA pathway.

It is known that the regulatory domain of SnRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid and osmotic stress signals controlling stomatal closure in *Arabidopsis* (Yoshida et al., 2006). It is worth noting that *CcSnRK2.6* was expressed only in leaves of the *C. canephora* clones after 72 h of ABA treatment. On the other hand, no expression was observed in *C. arabica* plants under control or ABA treatment. Regarding the *C. canephora* clones, it is worth noting that the D^S clone 22 present a basal regulation of this gene which was constant from control to ABA treatment.

These results are in accordance with the previous works in literature and also with the stomata measurements carried out during the hydroponic assay where there are significant differences among control and ABA 72 hours in the *C. canephora* D^T clone 14 but not in D^S clone 22. In the first 24h guard cells of *C. arabica* D^T I59 induced stomatal opening upon ABA stimulation and further promoted stomatal closure in the last 48h while no statistical differences were observed for Rubi. These evidences suggested that D^T clone 14 and I59 has been more efficient in the stomatal regulation under ABA exogenous treatment than D^S clone 22 or Rubi. Besides that, the absence of stomatal closure in response to ABA until 72h for Rubi is also in accordance with the delay in ABA signalling observed in gene expression analyses.

Finally, it is important to draw attention to the fact of *CcPYR1*, *CcPYL8b* and *CcSnRK2.7* and *CcSnRK2.11* were highly up-regulated in the D^T clone 14 and it suggest that they could act synergistically in the ABA pathway as key agents in a drought-tolerant response. All those evidences could be used to select molecular markers to improve genotypes selection in field.

CONCLUSION

Altogether, the results presented herein showed the expression of genes maintained or activated preferentially in response to ABA hormone. The ABA responses from *C. canephora* plants revealed to be different to *C. arabica* genotypes. In *C. canephora*, the D^T clone 14 presented higher expression for the *AHG2*, *AHG3*, *HAI* (*PP2Cs*) and *SnRK2.7*, *SnRK2.11* (*SnRK2s*) compared to the D^S clone 22 under control conditions which suggest the existence of the tripartite system (mode off) ready to be activated in the D^T plants. With ABA (24h) it was observed a higher and faster expression of *PYR1*, *PYL8b*, *SnRK2.7*, *SnRK2.11* concomitant with a drastic decrease for *AHG2*, *ABI* and *ABI2* showing an activation of tripartite system (mode on). On the other hand, the D^S clone 22 in response to ABA (24h and 72h) could not activate the synthesis of new ABA receptors or kinases, on the contrary, it activated the synthesis of *AHG2* gene which coding a phosphatase that negatively control ABA pathway. All this evidences support the phenotype differences (e.g. stomatal control) observed for drought tolerance between the D^T clone 14 and the D^S 22 suggesting that it could be consequence of the differences observed in the expression profiles of *PYL-PP2C-SnRK2* genes.

In *C. arabica*, it was clearly that the D^T I59 had a faster response to ABA stimuli compared to the D^S Rubi. With 24h it was observed that the D^T I59 up-expressed the *PYL8a*, *ABI1* and *2*, *AHG3*, *HAI*, *SnRK2.8* and *SnRK2.11* genes. The expressed phosphatases inhibit the activity of the kinases which could explain the absence of stomatal closure responses in leaves of I59 at 24h (mode off). Besides that, in both 24h and 72h was not possible detected the expression profiles of the tripartite system in I59 which could explain the stomatal closure at 72h, suggesting that some genes could be up-regulated between 24h and 72 h in *C. arabica* I59.

Regarding *C. canephora* and *C. arabica*, it was observed that *PYL8a*, *ABI1*, *HAB*, *SnRK2.8*, *SnRK2.11* genes were up-regulated in the D^T (clone 14 and I59) compared to D^S (clone 22 and Rubi) plants under control conditions. In response to ABA treatment, *PYR1* and *HAI* were up-regulated after 24h while *PYL9* and *SnRK2.8* after 72h. Considering the differences between species, it was showed that *SnRK2.6* gene was expressed at 72h only in *C. canephora* plants (clones 14 and 22). It was also observed that *CcPYL9* was up-regulated in the *C. canephora* (clones 14 and 22) and in *C. arabica* (I59) all those presented significant stomatal closure in response to exogenous ABA.

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GENERAL CONCLUSION AND PERSPECTIVES

The results presented in this work are one of the first that use the data generated by *C. canephora* sequencing, recently published, to analyze gene families such as those that codify proteins belonging to the tripartite system of ABA perception and signal transduction pathway. Comparing to studies developed in other species, our results showed for the first time the existence of duplication event in the *PYL* gene-family, notable for *CcPYL8*.

The results of expression analyses allowed us to confirm that the majority of the selected genes are functional in leaves and roots tissues. Similarly, several works have evidenced the importance of ABA tripartite system genes to fruit maturation highlighting the importance of further studies characterizing the *PYL* gene-family expression during coffee seeds development.

Despite the relevant information assessed with exogenous ABA experiment where genes expressed in response to this phytohormone presented distinguished regulation profile (mode on) in the D^T clone 14 and (mode off) in the D^S clone 22, a similar hydroponic test with different ABA concentration (lower) and number of plants (higher) in a different timepoint could be of interest. The evaluation of homoeologous gene expression in *C. arabica* subgenomes could provide useful information on this species plasticity to regulate ABA signaling and response pathways.

Even if our results did not present significative differences among clones regarding the amount of ABA in leaves and roots, it could be interesting to quantify this phytohormone under water deficit during a timecourse. Indeed, ABA quantification in plants with -3,0 MPA Ψ_{pd} value in stress condition (after 6 days watering withheld for the D^S clone 22, and between 12 and 15 days for the D^T clones, Marraccini et., 2011), did not allow to know if ABA content could variate in leaves and roots early after stress application. To verify that ABA metabolism

is not altered in the different *C. canephora* clones, it could be also interesting to test the gene expression of *CcNCED3* and *CcCYP707A1*, which are respectively involved in synthesis and catabolism of ABA. This work is also underway in the laboratory (Costa *et al.*, manuscript in preparation).

The results presented in this study confirm those previously obtained (Vieira *et al.*, 2013) which showed that drought tolerance response in *C. canephora* is a result of several correlated mechanisms rather than a single one. In addition, it would be interesting to search for single nucleotide polymorphisms (SNPs: *single-nucleotide polymorphisms* and indels: *INsertion*/*DELetion*) in the genes identified in this work, for example, in the genomes of D^T clones (14, 73 and 120) and D^S (22) of *C. canephora* since these are sequenced (AC Andrade, personal communication).

This research could be conducted both in the coding sequence, to search for proteins modifications in the tripartite system genes of D^T and D^S clones used in this work, and within their regulatory sequences (promoters) to verify the occurrence of sequence variations in *cis*-regulatory elements that could explain the different expression profiles observed for some genes in D^T and D^S clones, as has recently been observed for *CcDREB1D* gene of *C. canephora* (Alves *et al.*, submitted).

Finally, those genes with higher correlated drought-induced expression identified during this work (e.g *CcAHG2* and *CcSnRK2.2*) could be tested in other *C. canephora* drought tolerant and sensitive clones (Carneiro *et al.*, 2015) to find out if their expression profiles are kept. If that is the case, then one might consider using them as molecular markers in the coffee breeding programs for the generation of new drought tolerant varieties.