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**"Análisis de la función del gen para la MAPKKK YDA potencialmente
involucrada en desarrollo de floema"**

TESIS
Que presenta:

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"Analysis of the MAPKK kinase YODA gene function potentially involved in development of phloem."

THESIS

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ABBREVIATIONS

ABA	Absicic Acid
ACD	Asymmetric Cell Division
APL	Altered Phloem Development
AN3/GIF1	Angustifolia 3/ Growth Regulating Factor-interacting factor 1
ATHB	Arabidopsis thaliana Homeobox
ARF	Auxine Response Factor
BASL	Breaking of Asymmetry in the Stomatal Lineage
BES1	BRI1-EMS-suppressor 1
BIN2	Brassinoesteroid Insensitive 2
BDL	Bodenlos
bHLH	basic Helix-Loop-Helix
BL	Brassinolide
BNS	Basal Medium
BR	Brassinosteroid
BR1	Brassinosteroid Insensitive 1
BRL	Brassinosteroid Insensitive 1 like
BSU1	BR1 Suppresor 1
BZR1	brassinazole resistant1
Ca	Cambium
CA-YDA	Constitutively Active-YDA
CC	Companion Cell
CLE 41/44	CLAVATA3/Embryo Surrounding Region-related 41/44
CmPP16	*Cucurbita maxima* Phloem Protein 16
COP1	Constitutive Photomorphogenic 1
CPD	Constitutive Photomorphogenic Dwarf
CRY1	Cryptochrome 1
CT	Cycle treshold
DDB1	Damage DNA Binding 1
DET1	De- Etiolated 1
DMSO	Dimethylsulfoxide
dpi	Days post inoculation
DR5	Direct Repeat 5
DWF7	Dwarf7
EPF	Epidermal Factor
EPFL	Epidermal Factor-like
ER	Erecta
ERL	Erecta-like
FLP	Four Lips
FT	Flowering Locus T
Ft	Flow through
FTIP	Flowering Locus T- Interacting Protein
GA	Gibberellic Acid
GFP	Green Fluorescent Protein
GMC	Guard Mother Cell
GN	Gnom
GRAS	Gibberellin insensitive (GAI), Repressor of GA1-3 (RGA), SCARECROW(SCR)
GSK3	Glycogen synthase kinase 3
GUS	β-Glucuronidase
HBT	Hobbit

HR	Hypersensitive Response
IAA	Indol Acetic Acid
ICE1	Inducer of CFB Expression 1
IRX3	Irregular Xylem 3
JKD	Jackdaw
KAN	Kanadi
KIN	Kinetin
LB	Luria Bertani médium
LRR	Leucine-Rich Repeat
MatGAT	Matrix Global Alignment Tool
MAPK	Mitogen Activated Protein Kinases
MAPK	MAP Kinase
MKK	MAP Kinase Kinase
MAPKKK	MAP Kinase Kinase Kinase
MEK2	MAP/ERK Kinase 2
MMC	Meristemoid Mother Cell
MP	Monopteros
MS	Murashige- Skoog medium
NAC	No apical meristem (NAM), Arabidopsis thaliana activation factor (ATAF), Cup Shaped Cotyledon (CUC)
OPS	Octopus
ORF	Open Reading Frame
OX	Overexpression
PD	Plasmodesmata
Ph	Phloem
PHB	Phabulosa
PHYA/B	Phytochrome A/B
PHV	Phavoluta
PIN	PIN-FORMED
PLT	Plethora
PXY/TDR	Phloem Intercaled with xylem/ TDIF Receptor
pYDA	YDA promoter
QC	Quiescent Center
qRT-PCR	Quantitative real time reverse transcription polymerase chain reaction
RAM	Root Apical Meristem
REV/IFL1	Revoluta/Interfascicular 1
SA	Salicylic Acid
SAM	Shoot Apical Meristem
SHR	Short Root
SCR	Scarecrow
SCRM/SCRM2	Scream/Scream 2
SDD1	Stomatal Density Distributor 1
SE	Sieve Element
SETPHs	Sieve elements transcript promoters homologs
SLGC	Stomatal Lineage Ground Cell
SPA1	Supressor of PHYA-105 1
SPC	Stomatal Precursor cells
SPCH	Speechless
SSP	Short Suspensor
SUC2	Sucrose Transporter 2
TB	Terrific Broth medium

TDF1	tracheary Element Differentiation Inhibitory Factor
TF	Transcription Factor
TMM	Too Many Mouths
UBQ10	Polyubiquitin 10
Vb	Vascular bundle
VND6/7	Vascular-related NAC Domain 6/7
WOL	Wooden Leg
WOX	Wuschel-Related Homeobox
WT	Wild-type
XIP1	Xylem Intermixed with Phloem 1
Xy	Xylem
YDA	YODA
ZYMV	Zuccini Yellow Mosaic Virus

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ABSTRACT

YDA is a MAPKKK that controls asymmetric cell division in zygotes and stomata, as well as pedicel length, inflorescence architecture (Lukowitz *et al.,* 2004, Bergmann *et al.,* 2004, Meng *et al.,* 2012) and regulates cell division orientation plane in lateral roots (Smékalová *et al.*, 2014).*YDA* loss of function suppresses asymmetric divisions of the zygote, whereas in gain of function causes excessive elongation of the embryo making it difficult for functional studies in post-embryonic stages. Guard cell formation is negatively regulated by a MAPK pathway; thus, its activation leads to control over the number of these cells. The MAPK pathway in stomata requires other MAPKs such as MAPK3/6 and MKK4/5 and the GSK3 protein BIN2, which is a brassinosteroid-responsive protein that regulates YDA activity in this organ. YDA also plays a role in response to drought stress through control of stomatal density; AN3 regulates transcriptionally *YDA* to accomplish this function (Meng *et al.,* 2015)

Analysis of the *YDA* promoter showed its expression in phloem, which suggested a possible role for this protein in vascular tissue differentiation. We analyzed the role of YDA in this process in plants harboring non-lethal *YDA* alleles (*yda-1, emb71*) that display partial loss of function. These showed an alteration in vascular bundle shape; furthermore, a reduced number of cambium and phloem cells were observed in inflorescence stems. Treatment with exogenously added growth regulators revealed that *YDA* expression is induced by cytokinins and salicylic acid in seedlings, while in adult tissues brassinosteroids (BR), gibberellic acid (GA), auxin, abscisic acid (ABA) and salicylic acid (SA) alter *YDA* expression.

Since some elements of the stomatal MAPK pathway are also involved in development of other tissues and plant functions, it is possible that during evolution they were recruited for vascular tissue differentiation.

RESUMEN

YDA es una MAPKKK con función reportada en división asimétrica del cigoto, la formación de estomas, la longitud del pedicelo y la arquitectura de la inflorescencia (Lukowitz *et al*., 2004, Bergmann *et al*., 2004, Meng *et al*., 2012); además, controla el plano de orientación de las raíces laterales durante la división celular (Smékalová *et al*., 2014) La pérdida de función en *YDA* suprime las divisiones asimétricas del cigoto, mientras que en la ganancia de función se produce un alargamiento excesivo de la célula que va a generar el embrión haciendo difícil los estudios funcionales en etapas post-embrionarias. YDA se regula post-traduccionalmente por BIN2, la cual es una proteína de tipo GSK3 y está involucrada en la ruta de brasinoesteroides, en estomas. La activación la ruta de MAPKs, de la cual YDA forma parte, regula el número de células guarda y también involucra otras MAPKs, tales como MAPK3 / 6 y MKK4 / 5 YDA también se ha reportado en respuesta a estrés por sequía controlando la densidad de los estomas. AN3 regula la expresión de *YDA* para llevar a cabo esta función (Meng *et al*., 2015)

Análisis del promotor de *YDA* mostró su expresión en floema lo cual sugiere un posible papel de esta proteína en la diferenciación de tejido vascular. Se analizó el papel de YDA en este proceso utilizando plantas que presentan alelos *YDA* (*yda-1, emb71*) que muestran pérdida parcial de la función. Estos mostraron una alteración en la forma del haz vascular; además, se observó un número reducido de células cambium y floema en tallos de inflorescencia. El tratamiento con reguladores de crecimiento reveló que la expresión de *YDA* es inducida por citocininas y ácido salicílico en etapa de plántula, mientras que en tejidos adultos brasinoesteroides (BR), ácido giberélico (GA), auxinas, ácido abscísico (ABA) y ácido salicílico (SA) alteran la expresión *YDA*.

Debido que algunos elementos de la vía MAPK de estomas también están presentes otros tejidos y funciones de la planta, es posible que durante la evolución estas rutas hayan sido reclutadas para la controlar diferenciación de tejido vascular.

INTRODUCTION

1   INTRODUCTION

1.1   Symmetry breaking occurs first in the embryo

Animals and plants coordinate developmental processes to better adapt to their environment, although with striking differences, i.e. the establishment of the adult body in animals occurs during embryogenesis, whereas in plants the formation and patterning of adult organs occurs throughout post-embryonic stages, thus enabling a higher level of adaptation in plants. This is possible due to the symmetry breaking of the meristematic cells to generate new organs and tissues, in a process known as asymmetric cell division.

Asymmetric cell division is the process that generates specialized cells in multicellular organisms yielding diversity and patterning, as well as maintaining the stem cell pool. Asymmetric cell division requires many steps (Figure 1). First, specification of the cell or cells that will divide asymmetrically; this is followed by the establishment of asymmetry through localization of polar proteins, nuclear positioning and cytoplasm accumulation; then, the rearrangement of cellular components such as proteins, nucleic acids, cytoskeleton occurs before cell division, and finally the correct positioning of the cytokinesis machinery to establish the polarity of the cell and to give rise to asymmetric daughter cells (Paciorek and Bergmann, 2010; De Smet and Beeckman, 2011).
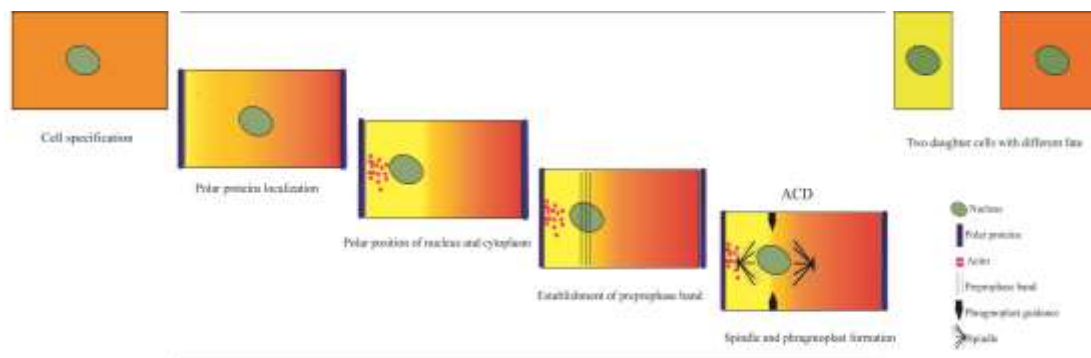
Figure 1. Asymmetric cell division process. Symmetry breaking is required for the establishment of polarity before start cell division to generate two different daughter cells

Before symmetry breaking occurs, a process known as polarity determination must take place. Endogenous signals as well as environmental cues determine the role of proteins and cellular components that establish polarity in different cells. Intrinsic factors determine the unequal features of daughter cells at birth while extrinsic factors determine the distinct fate of these cells due to differential post-division signaling and could act in combination to direct regulation of asymmetry (Petricka *et al.,* 2009; Abrash and Bergmann, 2009).

In plants, several factors determine the polarization of the cell from early stages of the embryo. In the model plant, *Arabidopsis thaliana*, PIN proteins and transcription factors (TFs) that determine division patterns in both apical and basal cells during the initial zygote division have been identified; also, hormones and other molecules that provide positional information have been reported (Mordhorst *et al.,* 1997; Petricka *et al.,* 2009; De Smet and Beeckman, 2011).

 The zygote is formed by the fusion of one ovule with one sperm cell (Mordhorst *et al.*, 1997). The first division of the apical cell is longitudinal; the cell divides until it reaches the eight-celled proembryo stage. The basal cell divides transversely until it has seven to nine cells, forming the suspensor that provides nutrients to the embryo.

 During early embryogenesis, apical-basal polarity is established. Indeed, one of the earliest steps during embryogenesis is the formation of the hypophysis from the uppermost cell of the suspensor, which is incorporated into the embryo generating the quiescent center (QC) of the root apical meristem (Yeung and Meinke, 1993; Mordhorst *et al.,* 1997; Lukowitz *et al.,* 2004; Petricka *et al.,* 2009; Paciorek and Bergmann, 2010; Zhang and Laux, 2011; De Smet and Beeckman, 2011; Petricka *et al.,* 2012).

The organs of the basic plant body are defined by asymmetric divisions during embryogenesis (Lukowitz *et al.,* 2004). The apical and the basal polar axes grow longitudinally and the outer and inner axes radially. During this process the shoot apical meristem (SAM) and the root apical meristem (RAM) are established (Mordhorst *et al.,* 1997; Wolters and Jürgens, 2009; Lau *et al.,* 2010; Dettmer and Friml, 2011).

Three primary meristems for radial growth exists: the protoderm, which precedes the epidermis during the primary growth and cork periderm during secondary growth; the fundamental or ground tissue that consists of parenchyma, collenchyma and sclerenchyma; and the vascular cambium that generates primary and secondary xylem and phloem (Mordhorst *et al.,* 1997; Wolters and Jürgens, 2009).

The family of *PIN* genes mediates auxin transport by a gradient determining the polarity of the cell. Localization of *PIN7* and the auxin response factor (ARF) *DR5* shows that *PIN7* is expressed on the apical membrane of suspensor cells while *DR5* is found in the apical cell lineage suggesting that auxin is transported acropetally during embryogenesis (Petricka *et al.,* 2009).

After zygote division, auxin accumulates in the apical cell. PIN7 acts in auxin transport from the basal to the apical cell to form the proembryo. During the globular stage, PIN7 mediates auxin transport out of the embryo and is reversed within basal cells. Proembryo development is impaired in *pin7* mutants, similar to *gnom* mutants (*EMB30* allele), both required for directional trafficking of PIN1 protein (Müller *et al.,* 1998; Steinman *et al.,* 1999; Friml *et al.,* 2002; Friml *et al.,* 2003; Lukowitz, 2004; Blilou *et al.,* 2005).

PIN1 localizes to provascular cells and determines the root pole during the globular stage of embryo development. The initial expression of *PIN4* is at the basal pole of the embryo supporting activity of PIN1 and PIN7 (Friml *et al.* 2003).

Early embryo development depends on different factors such as the transcription factor MP and its antagonist BDL. The *mp* and *bdl* mutants fail to initiate the hypophysis although their expression is limited to the proembryo (Berleth and Jürgens, 1993; Hamann *et al.,* 1999). Members of the *WOX* gene family are also expressed during early embryogenesis. *WOX2 and WOX8* are expressed in the zygote; after the first division *WOX2* expression is restricted to the apical cell while *WOX8* is expressed in the basal lineage as well as its closest homolog *WOX9.* When the proembryo is formed other WOX proteins, WUS and WOX5, trigger signals for maintenance of the shoot and root stem pool (Breuninger *et al.,* 2008).

The *wox8* and *wox9* mutants do not express *WOX2,* with evident defects during zygote elongation, becoming more symmetric (Breuninger *et al.,* 2008). This phenotype is similar to the

YDA mutants. YDA is a MAPKKK involved in suspensor cell formation (Lukowitz *et al.,* 2004). YDA and WOX are necessary for the establishment of zygote polarity, despite the fact that they do not seem to participate in the same pathway (Breuninger *et al.,* 2008).

1.2 Mechanisms for Asymmetric Cell Division During Stomatal Differentiation

Asymmetric cell division has been thoroughly studied in stomata. These organs are specialized pores in leaves and stems formed by two guard cells with a pore in the center. Stomata control the loss of water vapor and are required for gaseous exchange of carbon dioxide during photosynthesis, and oxygen, in respiration. In Arabidopsis the regular distribution of stomata consists of at least one pavement cell between two guard cells, allowing ion exchange between them (Geisler 2000; Nadeau and Sack 2002; Bergmann *et al.,* 2004; Barton, 2007).

Stomata development goes through several transition stages. A protodermal cell differentiates into a meristemoid mother cell (MMC). This then undergoes asymmetric cell division to generate a small cell (the meristemoid) and a large cell that differentiates into a pavement cell. The transcription factor SPCH is involved in MMC formation and entry into asymmetric division as well as spacing divisions. Plants with *SPCH* loss-of-function display joined pavement cells, whereas its ectopic expression causes excessive division of pavement cells (Ohashi-Ito and Bergmann, 2006; Barton, 2007; Bergmann and Sack, 2007*;* Lampard *et al.,* 2008; Petricka *et al.,* 2009; Abrash and Bergmann, 2009; Lau and Bergmann, 2012). The transcription factor MUTE promotes meristemoid transition to guard mother cell (GMC). Loss of function of MUTE results in a rosette pattern of cells surrounding a small arrested meristemoid-like cell, while its overexpression generates excess stomata. FAMA is another transcription factor that controls the differentiation of GMC to guard cells (GC); FRL and MYB88 proteins are also required for this process. Overexpression of FAMA generates ectopic guard cells (Ohashi-Ito and Bergmann*,* 2006; Barton, 2007; Bergmann and Sack, 2007; Lampard *et al.,* 2008; Lau and Bergmann, 2012).

ER, ERL and TMM constitute the main receptor family in plants, and regulate stomatal development. ER receptors display redundant functions; they are involved in cell proliferation, and are expressed in the protoderm, but prevent symmetry breaking in the MMC (Sphak *et al.,*

2005; Ingram, 2005; Lau and Bergman, 2012). ER acts synergistically with other ERL promoting proliferation of the meristemoid cell while repressing its differentiation into a guard mother cell (Ingram *et al.,* 2005). Stomatal patterning also requires TMM action. TMM acts upstream in the stomatal development pathway and is expressed in both daughter cells from MMC. TMM promotes the spacing divisions and regulates, negatively, cell proliferation (Sack, 2004; Ingram, 2005). TMM produces a slight defect in leaves but in stems stomata are absent, its function being epistatic to ER, ERL2 and partially to ERL1 in stems (Sack, 2004; Ingram, 2005).

SDD1 is a subtilisin-like protease expressed in the small cell generated from the MMC and is a putative signal upstream in the stomatal development pathway (Sack, 2004). Similarly, the family of secreted peptides EPFL are involved in stomatal development and plant growth; they are recognized by LRR receptors such as ER and TMM that, surprisingly, appear to act independently of SDD1 (Hunt *et al.,* 2010; Lee *et al.,* 2012; Lau and Bergmann, 2012). EPF1 and 2 interact with ERL1 to inhibit stomatal development; TMM promotes this interaction, thus inhibiting the activation of ERF. Another peptide, EPFL9, or STOMAGEN, is expressed in the mesophyll and promotes stomata formation counteracting EPF1 and EPF2 (Hunt *et al.,* 2010; Sugano *et al.,* 2010; Lee *et al.,* 2012; Lau and Bergman, 2012).

Other genes involved in stomata differentiation are *PEAPOD1* and *2*, which control extra cell divisions in the leaf epidermis as well as cell division associated with the vasculature (Barton, 2007).

Several polarity regulators have been identified in asymmetric cell division of stomata, such as BASL, which is expressed in MMC, meristemoid and stomatal lineage ground cells (SLGC). Its role is determining the axes before division; interestingly, the subcellular localization of this protein determines the fate of the daughter cells, i.e., nuclear localization determines guard cell differentiation, whereas peripheral accumulation leads to differentiation into pavement cell (Fu, 2002; Dong *et al.,* 2009; Lau and Bergman, 2012). It has been suggested that this asymmetrical distribution is determined by ROP proteins, which regulate polar expansion in pavement cells through reorganization of the actin cytoskeleton, resulting in polarized domains (Dong *et al.,* 2009).

Another factor involved in determining asymmetry is POLAR, which localizes to the larger cell and depends on BASL activity. POLAR is also eventually distributed unequally between stomatal precursor cells; its level decreases in the larger cell while increasing in the opposite meristemoid cell (Pillitteri *et al.,* 2011).

Two additional paralogous proteins, SCRM and SCRM2, as well as their close homolog ICE1, are involved in stomatal cell fate; indeed, gain of function in SCRM promotes stomata formation (Kanaoka *et al.,* 2008). SCRM and SCRM2/ICE1 are bHLH leucine zipper proteins, which are interacting partners of SPCH, FAMA and MUTE; they possibly form heterodimers to regulate their downstream targets. SCRM may lie upstream of FAMA and MUTE but is epistatic to SPCH (Kanaoka *et al.,* 2008).

ICE1 expression is induced by cold stress and is strong in GMC and meristemoids, and as with SCRM, depends of SPCH activity in stomata lineage. Its protein accumulation pattern is quite similar to that of SCRM2, except that is not expressed in mature GC. Both proteins are involved in directing progression into asymmetric division of stomata (Kanaoka, *et al.,* 2008).

1.3   Symmetry Breaking is Required for Root Development

During embryo development, the hypophysis is formed from the uppermost suspensor cell. The asymmetric cell division of the hypophysis then generates the different structures of the RAM, an upper lens-shaped cell which gives rise to the QC and a lower basal cell which forms the columella stem cells and columella (Petricka *et al.,* 2009, De Smet and Beeckman, 2011, Petricka *et al.,* 2012). The QC is the stem cell pool of the RAM and the cells on the shootward and lateral regions give rise to concentric layers of root tissue: epidermis, cortex, endodermis, pericycle and vasculature. The endodermis and cortex originate by asymmetric divisions of the cortical/endodermis initial cells. The first division is anticlinal and produces one initial cell and one daughter cell. The daughter cell then divides periclinally to give rise to the endodermis and the cells of the cortex lineage (Di Laurenzio *et al.,* 1996).

The auxin response factor MP and its repressor, an Aux/IAA protein, BDL are involved in hypophysis specification, although their expression is found in provascular cells adjacent to this tissue (Berleth and Jürgens 1993; Hamann *et al.,* 1999). When MP is derepressed, PIN1 is

upregulated in provascular cells adjacent to the hypophysis, allowing auxin transport and specifying its formation.

Another gene involved in root development is *HBT.* Mutants in this gene show defects in hypophysis formation precluding the generation of a QC and columella (Willensen *et al.*, 1998).

Two pathways are involved in specification of the QC identity: the *PLT* pathway and the *SHR-SCR* pathway (Di Laurenzio *et al.,* 1996; Helariutta *et al.,* 2000; Aida *et al.,* 2004). Two *PLT* genes are required for embryonic specification of the QC cells, *PLT1* and *PLT2*; these are expressed in the stem cell pool and its transcription depends on ARFs such as MP and NPH4 (Aida *et al.,* 2004).

SCR and SHR belong to the GRAS family and regulate the radial patterning in the root (Di Laurenzio *et al.,* 1996, Helariutta *et al.,* 2000). The *scr* mutants fail in the periclinal division of the cortical/endodermis daughter cell, resulting in a single layer with mixed identity (cortical and endodermis features). The expression of SCR occurs in cortical/endodermis initial cells and then is restricted to the endodermis cell lineage (Di Laurenzio *et al.,* 1996). SHR is a non-cell-autonomous protein, whose expression occurs in the stele (Helariutta *et al.,* 2000). SHR moves into the ground tissue and interacts with SCR in the nucleus to regulate the transcription of SCR and other target genes involved in symmetry breaking. It was suggested that an increase in SCR levels blocks the SHR movement resulting in a positive feedback (Cui *et al.,* 2007).

In post-embryonic stages, small, secreted peptides called root-meristem growth factors promote the maintenance of the stem cell pool by post transcriptional regulation of PLT proteins and JKD, a transcription factor required for the expression of SCR in the QC (Petricka *et al.,* 2012).

1.4   Vascular Tissue Development

The plant vasculature is a complex system that interconnects the whole plant. It provides plant body support, and transport of minerals, photoassimilates and other macromolecules throughout the plant. This extended network is formed by two different types of tissues: xylem and phloem; both are comprised of specialized cells, the tracheary elements and vessels of xylem, and companion cells and sieve elements of phloem.

The ontogeny of the vascular tissue is regulated by intrinsic and extrinsic factor determining the patterning that will give rise to the vascular cells in the different organs of the plant. During embryonic development meristematic cells start to differentiate along the root-hypocotyl axis (Sieburth and Deyholos 2006). Vascular tissue formation occurs in two different phases: primary and secondary growth. Primary growth is the increase in cell length and maturation, whereas secondary growth gives rise to an increase in girth. Both occur in most gymnosperms as well as in dicotyledonous angiosperms. Monocotyledonous plants usually lack secondary growth; instead, there is a thickening growth resulting from parenchyma enlargement (Scarpella and Meijer, 2004).

The vascular system is formed continuously from root to stem, during primary growth, in spite of the different arrangement between these two organs (Altamura *et al.,* 2001). In the early globular stage, the procambium is formed and at the end of this stage cells start to divide periclinally giving rise to the pericycle and vascular primordium. After germination, the pre-procambium differentiates into procambial tissue to form primary xylem and phloem (Elo, 2009). In leaves, the provascular cells present a specific positional pattern and become procambial strands to generate the network of vascular bundles (Sieburth and Deyholos, 2006). Vascular bundles are formed during primary growth, while vascular patterning, including the formation of vascular rings, occurs during secondary growth.

Secondary growth starts with periclinal asymmetric divisions of the meristematic cells presents in the pericycle in roots, interfascicular regions in shoots and procambial cells in shoots and roots to give rise to the vascular cambium. These meristematic cells retain their location between the xylem and phloem within the vascular bundles, and are necessary for radial growth of stems and roots and regulating the lateral growth of interfascicular regions between vascular bundles. The cambial activity of shoots starts in the primary vascular bundles and proceeds then in the interfascicular regions (Elo *et al,* 2009), a mixture of different cells at the end of the secondary growth that, in a mature plant, have primary and secondary components that are lignified and contribute to the stem stiffness (Altamura *et al.,* 2001). The secondary vascular growth in the stem of dicots is associated with the patterning present in roots but changes in branches since the pattern of the vascular tissue is defined in an organ-specific manner (Altamura *et al.,* 2001)

1.5   Vascular Tissue Differentiation Involves Hormone Signaling

The chemical signals involved in the process of vascular organization and differentiation have not yet been elucidated in detail, although several key proteins involved in auxin transport from the shoot apical meristem and young leaves to older tissues appear to be crucial for vascular differentiation. This is the case of some genes such as *MP, PIN1* and *GNOM* (Okada *et al.*, 1991; Berleth and Jürgens, 1993; Hardtke *et al.*, 1998*;* Steinman *et al.,* 1999; Ye, 2002).

Auxin is the main regulator of xylem development; it directs anticlinal divisions producing the vascular cambium (Elo *et al.,* 2009), and, at high concentrations, causes differentiation of short fibers and their thick secondary walls (Zhong *et al.*, 1997). Gibberellins also influence vascular tissue development, in synergism with auxins. Cytokinins stimulate cell proliferation through cell cycle regulation, and, thus, its role in vascular differentiation is through inhibition, as indicated by some reports (Mahönen *et al.*, 2006). Studies in *Populus* showed that ethylene stimulates radial growth by increasing secondary xylem formation (Love *et al.,* 2009) and that the auxin pathway induces ethylene biosynthesis resulting in wood formation (Nilsson *et al.* 2008). BR were identified in the *Pinus* cambial zone, possibly regulating meristem activity (Kim *et al.,* 1990). Mutants in BR receptors or biosynthetic genes produce excessive secondary phloem instead of secondary xylem and result in flowering delay (Caño-Delgado *et al.,* 2004; Domagalska *et al.,* 2007). Xylem formation is promoted in *Zinnia* explants by brassinosteroids, suggesting an important role in xylem development (Fukuda, 1997). In *Arabidopsis*, mutants in the *CPD* and *DWF*7 genes involved in BR synthesis show defects in vascular differentiation (Szekeres *et al.*, 1996; Choe *et al.*, 1999).

BRL1, *BRL2*, and *BRL3* are genes with high similarity with *BRI1*, a brassinosteroid receptor gene (Caño-Delgado *et al.,* 2004). *BRI1* and *BRL1* double mutants display an increased number of phloem cells and decrease in xylem cells. This suggests that BRL1 and BRL3 are novel BR receptors that function specifically in provascular differentiation to maintain proper xylem:phloem ratios. Indeed, mutants in BR receptor or biosynthetic genes produce excessive secondary phloem instead of secondary xylem and exhibit flowering delay (Caño-Delgado *et al.*, 2004; Elo

et al., 2009). BR signaling in procambial cells promotes xylem differentiation, while repressing phloem formation (Caño-Delgado *et al.,* 2004).

Phosphoinositides may be involved in signaling during vascular differentiation because they have been found in procambium cells (Ettlinger *et al.,* 1988; Elge *et al.,* 2001; Ye *et al.*, 2002). Another indirect evidence in support of this notion is provided by the fact that auxin promotes phosphoinositide synthesis (Ettlinger *et al.,* 1988).

1.6   Two Gene Families Control Vascular Patterning

Vascular tissues are arranged in vascular bundles around the stem periphery. The xylem develops on the adaxial or internal pole whereas the phloem forms on the abaxial or external pole on the vascular bundles (Dinneny and Yanofsky, 2004). Two families of genes have been reported as responsible for determining adaxial and abaxial identity, the HD-ZIP III and KANADI (Bowman *et al.*, 2002).

The HD-ZIP III proteins determine differentiation on the adaxial pole, whereas the *KANADI* family of genes control the abaxial identity. Mutants in both HD ZIP III and *KANADI* genes show defects in vascular tissue on leaves, although they also function in vascular tissue of stems (Emery *et al.,* 2003) (Figure 2). The III HD-ZIP gene family member *ATHB8* is expressed specifically in procambium precursor and procambial cells and is also a direct target of MP. Other proteins of this family, such as ATHB15, PHV, PHB and REV / IFL1 are possibly regulated by MP since the expression of these genes is reduced in MP mutants (Baima *et al.,* 2001; Emery *et al.,* 2003; Dinneny and Yanosfky, 2004; Carlsbecker and Helariutta, 2005; Donner *et al.,* 2009; Ohashi-ito and Fukuda, 2010;). Alternatively, defects in the *IFL1* gene, an HD-ZIP family member, leads to an abnormal distribution of the interfascicular fibers in these arcs (Zhong *et al.*, 1997, Zhong *et al.*, 1999).

Miss-expression of members of the KANADI family results in defects in vascular development. For instance, in the triple mutant *kan1, kan2, kan3* the xylem surrounds phloem in the stem, similar to when the *REV* dominant allele is expressed (Dinneny and Yanosfky, 2004).

No defects are observed in VND7 and VND6 mutants but their ectopic expression causes cells as diverse as guard cells and root endodermis to re-differentiate into those resembling

protoxylem (VND7) or metaxylem (VND6) tracheary elements (Bonke *et al.*, 2003; Kubo *et al.*, 2005; Sieburth and Deyholos, 2006; Yamaguchi *et al.*, 2008; Ohashi-Ito *et al.*, 2010; Yamaguchi *et al.*, 2011).
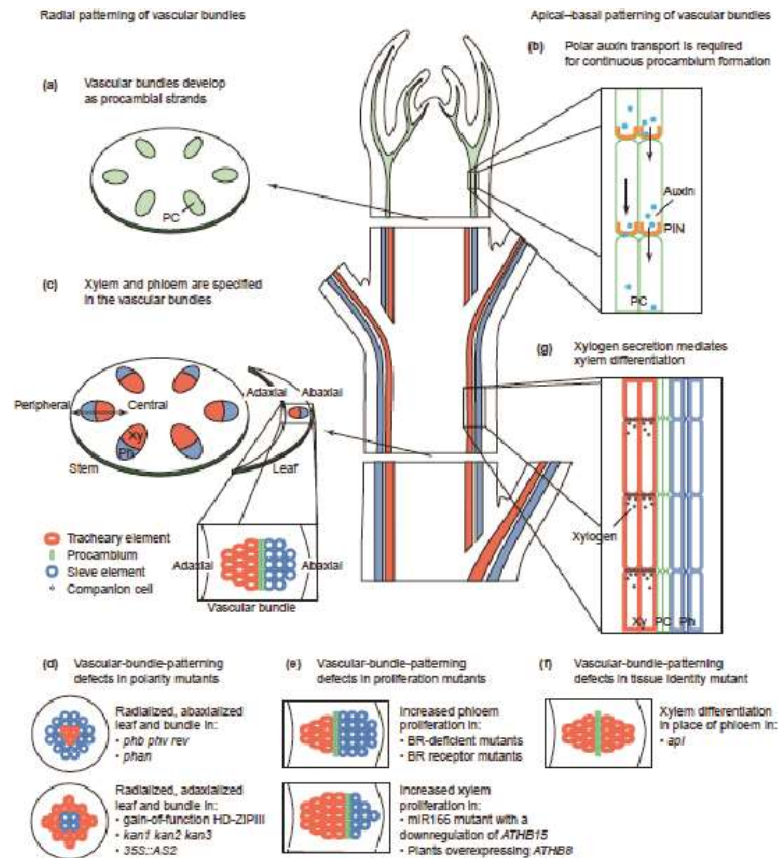
Figure 2. Representation of vascular bundle patterning regulated by HD-ZIP III and *KANADI* genes. Image from Carlsbecker and Helariutta (2005).

1.7   Phloem Fate Determination

Once a cell has been determined to become phloem, a maturation process that involves asymmetric cell division has to occur to generate the two cell types that form the functional phloem, the companion cell and the sieve element. Anticlinal divisions of a phloem mother cell give rise to elongated, enucleate cells, the sieve elements, which undergo organellar and cytoplasmic rearrangements to function as conducting cells; while two periclinal divisions

produce companion cells, which at maturity will provide cellular support for their neighboring enucleate sieve elements (Figure 3) (Bonke *et al.,* 2003; Elo *et al.,* 2009).
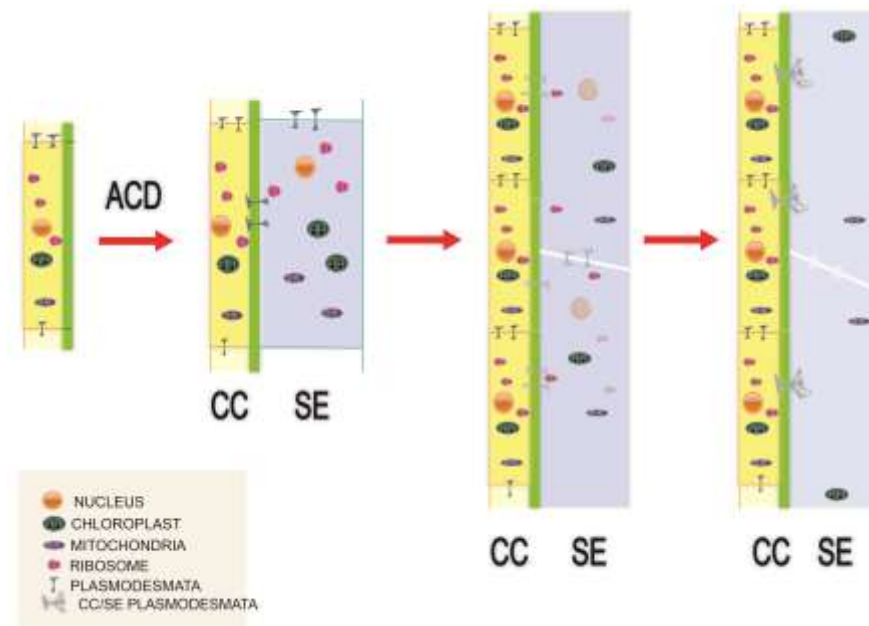
Figure 3. Phloem development representation. Precursor cell have asymmetric cell division to generate two daughter distinct cells CC and SE. (CC: companion cell; SE: sieve element). Then CC continues dividing symmetrically while SE elongates and lose some organelles. Endoplasmic reticulum and some ribosomes continue associate to CC/SE plasmodesmata while sieve plate is formed. Reproduced from Xoconostle-Cazares, Martínez-Navarro and Ruiz-Medrano, Phloem Long-Distance Trafficking of RNAs and Proteins; Encyclopedia Life Sciences, Elsevier (in press).

The receptor PXY/TDR controls cell orientation in procambium, via WOX4 activation, in turn involved in maintaining the procambium pool and inhibiting the auxin efflux carrier PIN1 (Fisher and Turner 2007). Secreted peptides such as TDF1, encoded by the *CLE41/44* gene and originating in phloem, interact with PXY (Hirakawa *et al.,* 2010). Another receptor that may have a role in vascular development is a leucine-rich receptor-like kinase termed XIP1. Histochemical analysis of the *xip1* mutants revealed abnormal lignified phloem cells, caused possibly by defects during phloem to xylem cell fate transformation, or as a result of alteration of cambium activity, suggesting a possible XIP1-mediated feedback with the TDF1-PXY-WOX4 pathway, regulating cambium proliferation and phloem cell differentiation (Bryan *et al.,* 2012).

Genes that have direct effects on vascular cell identity have been identified, such as *APL/ FE, VND6* and *VND7* (Bonke *et al.,* 2003; Ohashi-Ito *et al.,* 2010). The APL mutants fail to develop phloem sieve elements or companion cells, forming tracheary elements instead. Recently it has been found that APL controls *FT* transcription and therefore regulates flowering (Abe *et al.,* 2015). An additional gene is *WOL*, which is necessary for periclinal cell divisions in the root meristem that produces phloem cells (Scheres *et al.,* 1996).

OPS is a differentiation enhancer whose polar localization on the membrane determines phloem fate (Truernit *et al.,* 2012). This protein is expressed in provascular cells to specify differentiation and is restricted to the phloem lineage. The *ops* mutants display discontinuous differentiation in phloem, and when overexpressed, results in premature phloem differentiation. This suggests that OPS has a similar role to the xylogen in specifying the cells that will become protophloem; moreover, its localization in polar membranes determine vascular patterning (Truernit *et al.,* 2012). The GSK3 BIN2 is inhibited in the presence of BR in stomata (Kim *et al.,* 2012), although OPS is a positive regulator of BR response allowing the expression of key BR response transcription factors, such as BZR1 and BES1, by negative regulation of BIN2 during phloem differentiation (Anne *et al.,* 2015).

Evidently, while the role of some genes in vascular development has been elucidated, the full details of these pathways remain to be established. Clearly, more information is needed to identify common elements shared among routes within the different plant tissues and to find major regulators of the specific differentiation processes.

1.8 The MAPKKK YDA acts during Early Embryogenesis, Stomata development and inflorescence architecture

In Arabidopsis there are approximately 20 MAPKs, 10 MAPKKs and 80 MAPKKKs (MAPK Group 2002; Suzuki and Machida, 2008; Lau and Bergman, 2012). The number of MAPKs in plants is greatly expanded compared with their animal counterparts; this complexity could suggest tissue specificity and may also reflect cross-talk between different MAPK pathways, as well as redundancy, leading to a more fine-tuned regulation.

YDA MAPKKK has a role in controlling asymmetry in the zygote. Loss of function of YDA suppresses the asymmetric division in this cell, leading it only to embryonic fate. Gain of function mutants show excessive elongation, forming an exaggerated suspensor and suppressing embryo formation. This suggests that YDA activation promotes the asymmetry in the zygote (Lukowitz *et al.,* 2004).

YDA null-alleles are lethal during early embryo development; however, non-lethal alleles have been obtained in Arabidopsis, such as *yda-1* and *yda-2* that can advance through this developmental stage (Lukowitz *et al*., 2004). These plants exhibit a dwarfing phenotype, compressed shoots, sterile flowers and small rosettes. Root development delay and alterations in basipetal auxin transport in embryos were also observed. The YDA pathway that regulates embryogenesis seems to be controlled by SHORT SUSPENSOR, which is an interleukin-1 receptor-like associated kinase (Bayer *et al*., 2009).

Analysis of the epidermis in cotyledons and hypocotyl of *YDA* mutants showed overproduction of normal stomata, suggesting that YDA affects the frequency of divisions resulting in the regulation of stomatal density (Bergmann *et al.,* 2004, Sack, 2004,). Comparing the *YDA* phenotype with those of the *fama, mute* and *spch* mutants suggests that YDA acts upstream, inhibiting SPCH and thus stomata formation.

YDA regulates a large array of downstream genes, as revealed by the analysis of *YDA* mutants in which the expression profile of 1800 genes was altered (Bergmann *et al.,* 2004). Among these there were several involved in signaling and guard cell specification.
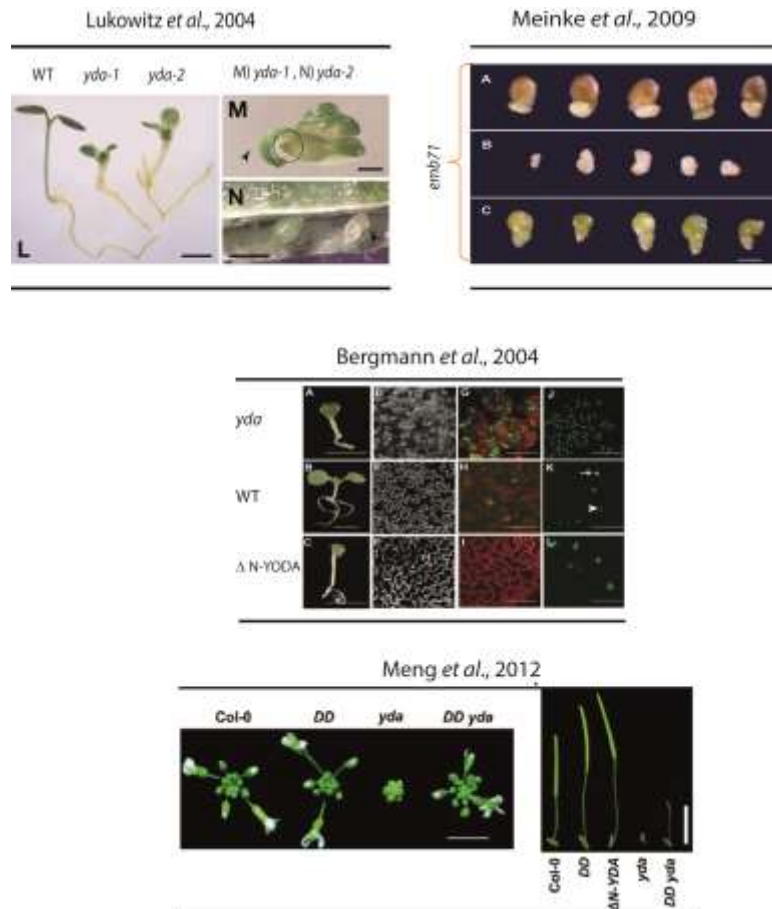
Figure 4. YDA acts in regulating embryo development, stomata formation and inflorescence architecture. Modified from Lukowitz et al.. 2004; Bergmann et al., 2004; Meinke et al.; 2009, Meng et al., 2012.

Several proteins have been identified that participate in the YDA pathway or specifically interact with it. Complementation assays using constitutively active YDA in *tmm* and *sdd1* backgrounds showed phenotype rescue, indicating that YDA acts downstream of these proteins; this is in contrast to *four lips* mutants, where YDA had no effect (Bergmann, *et al.,* 2004). Also in contrast with *four lips* mutants, *YDA* mutant plants develop GMC with microtubules radially oriented similar to mature guard cells, suggesting that *YDA* acts before the specification of the GMC (Bergmann *et al.,* 2004).

YDA has been also reported to regulate inflorescence morphogenesis, as the ER receptor and other MAPKs that had been described to act downstream of YDA the stomata pathway (MAPK3/5, MKK4/5) (Meng *et al.,* 2012, Uchida *et al.,* 2012, Bemis *et al.,* 2013). ER and the MAPKs produces short pedicels by regulating cortex cells formation but YDA mutants results in more severe phenotype generating clustered inflorescences with shorter pedicels and siliques. As constitutively active YDA (CA-YDA) rescues the phenotype of ER mutants it can be concluded that both are acting in the same pathway as in the stomata development (Meng *et al.,* 2012)

1.9   Other MAPKs Involved in Stomatal Development and inflorescence morphogenesis

MKK4/5, MPK3, and MPK6 have been linked to responses to environmental stimuli and are part of the *YDA* pathway. Loss of function of these MAPKs results in stomatal clusters whereas their gain of function disrupts stomatal formation, essentially as in *YDA* mutants (Wang *et al.,* 2007). MKK4 and MKK5 are linked to stress response signaling (Asai *et al,* 2002) and have similar functions in this process (Yang *et al.,* 2001). The effect of MKK4/ MKK5 silencing suggests a redundant role in stomatal development (Wang *et al.,* 2007).

Inducible complementation assays for MEK2 in *YDA* null mutants showed a decrease in stomatal clusters, thus rescuing the phenotype (Wang *et al.,* 2007). This suggests that YDA acts upstream of MKK4 and MKK5 in several developmental pathways because YDA effects are similar to those displayed by MKK4, MKK5 and MEK2. Additionally, YDA enhances the phosphorylation of MAPK3/6 indicating that they act in the same pathway (Wang *et al.,* 2007). It is known that environmental stimuli have effects on stomatal density; indeed, MKK4/5 have been shown to be involved in stress response, integrating environmental changes with stomatal formation.

MAPK3/6 and MKK4/5 also act in pedicel elongation. Single mutants of these MAPKs causes no substantial effect on the phenotype; however double mutants are able to emulate the ER phenotype, while constitutively active MKK4/5 and NtMek2 (ortholog in tobacco of MKK4/5) can rescue the ER mutant (Meng *et al.,* 2012).

1.10 YDA activity depends of biotic and abiotic factors

Stomatal density is also regulated by light. The COP1 RING3 ubiquitin ligase is a photomorphogenic repressor and is regulated by several components of light signaling, such as CRY1, CRY2, SPA1, and the CDD complex (COP10, DDB1, DET1), PHYA and PHYB. The *cop1* mutants present a similar phenotype to that presented in *YDA* mutants. COP1 acts in parallel with TMM but upstream of YDA, MUTE, and FAMA, respectively (Kang *et al.,* 2009, Lau and Bergmann, 2012).

The effect of hormones on stomatal density has been observed. For instance, mutants in brassinosteroid-regulated genes, such as *BRI1, BZR1, BSU1* and *BIN2*, show phenotypes similar to those observed with mutants in stomatal development genes (Kim *et al.,* 2012). Complementation and interaction assays showed that YDA activity depends on components from the brassinosteroid-response pathway. YDA possesses a GSK3- regulation domain, which is phosphorylated to repress its activity. BIN2 is a member of the GSK3 protein family, which is constitutively active in the absence of brassinosteroids and is inhibited when the hormone is perceived (Kim *et al.,* 2012; Casson and Hetherington, 2012; Lau and Bergmann, 2012).

Further evidence supporting that the brassinosteroid-responsive pathway regulates YDA comes from the fact that *bin2* mutants allow the phosphorylation of the known targets of YDA,MAPK4/5 and MPK3/6, which suggesting that YDA and SPCH are both regulated by BIN2. This has led to a proposal for a new stomatal regulation pathway (Kim *et al.,* 2012).

Other studies in roots showed that YDA and MAPK6 regulate lateral root formation by the upregulation of auxins. YDA and MAPK6 also affect cell division plane orientation. In the *yda-1* mutant, expansion delay by the phragmoplast is observed, and agravitropism and a twisting phenotype occurs in roots of CA-YDA; this phenotype is emulated by addition of auxinol (Smékalová *et al.,* 2014).

AN3 is a GRF-interacting factor essential for regulation of the drought tolerance response. AN3 binds to YDA promoter inactivating its transcription to control stomata density and transpiration as well as root architecture. Due that CA-YDA reduces high stomata number produces by AN3

mutation, it is likely that AN3 acts upstream of YDA to control stomata development to avoid water lost (Meng *et al.,* 2015).

1.11 Role of YDA in Vascular Development

The PXY and XIP1 receptors belong to the same family as ER and TMM which act upstream of YDA in the stomatal pathway. PXY is expressed in cambium cells and its activity is controlled by a peptide that originates in phloem cells, TDF1, as described before (Hirakawa *et al.,* 2010). It is thus likely that YDA functions in vascular development.

Members of the WOX protein family show a remarkable division of labor. Indeed, the homeoproteins WOX2, WOX8 and WOX9 are expressed in the zygote to maintain the stem cell pool, while WOX4 is also present in procambium cells to promote their proliferation. WOX4 is activated by PXY and controls PIN1 action to inhibit differentiation. Although the exact role of XIP1 is not clear, it be could be involved in repressing proliferation and promoting differentiation (Fisher and Turner, 2007; Breuninger *et al.,* 2008; Bryan *et al.,* 2012).

YDA activity is regulated by a GSK3-like protein (BIN2), in stomata, which is inhibited when BR1 receptors are activated by brassinosteroids (Kim *et al.,* 2012). Evidence is lacking regarding BIN2 activity in vascular tissue or whether it has a role in phloem or xylem formation; however, it is possible that a similar pathway controls vascular tissue patterning in the presence of this hormone, as has been reported for the stomatal pathway, since brassinosteroids are also involved in vascular tissue patterning (Caño-Delgado *et al.,* 2004).

The YDA targets, MAPK3/6 and MKK4/5 have redundant activity and could be implicated in developmental processes as well as in abiotic or biotic stress responses (Wang *et al.,* 2007). Therefore, known targets of YDA could be expected to be present in vascular tissue.

The fact that some genes involved in stomata development are also required during embryogenesis, and, furthermore, also possibly involved in vascular tissue differentiation, suggests that individual components of developmental pathways remain linked in plants. More studies are required to elucidate all the components involved in symmetry-breaking in plants, as

well as to determine the genes that regulate the asymmetric cell division that generate xylem and phloem.

A function for YDA in vascular tissue development is further supported by the fact that the YDA promoter is active in leaf epidermal, stomatal and vascular tissues (Bergmann *et al*., 2004; Ruiz-Medrano *et al*., 2011). Furthermore, post-transcriptional gene silencing of YDA in Arabidopsis showed changes in vascular tissue arrangement and inflorescence stem shape (Martínez-Navarro 2011, Master thesis)

As indicated above, current evidence indicates an important role of YDA in embryogenesis and stomatal differentiation (Bergmann *et al.,* 2004; Lukowitz *et al.,* 2004; Meinke *et al.,* 2009). Also, we have described how stomatal differentiation is linked to other pathways involved in response to light and brassinosteroid regulation. Additionally, the activation of the stress-related MPK3/6 MAP kinases by YDA points to a role of YDA in biotic and and abiotic stress responses (Wang *et al.,* 2007, Kang 2009, Kim *et al.,* 2012). Furthermore, recently, an important function for YDA in pathogen response has been reported, essentially as the other MAPKs downstream of it, indicating that the same developmental pathway is shared by pathogen-triggered immunity (Sopeña-Torres 2015, PhD thesis).

HYPOTHESIS

2 HYPOTHESIS

- The MAPKKK YDA plays an important role in the differentiation of the vascular tissue, and particularly in the phloem.

OBJECTIVES

3   OBJECTIVES

- Determine the role of AtYDA in vascular tissue differentiation in *Arabidopsis thaliana.*

3.1   Specific objectives

- Elucidation of the expression pattern of *YDA* in Arabidopsis grown under different growth regulators.
- Analysis of the expression pattern of *YDA* in different tissues, particularly in the vasculature.
- Analysis of vascular tissue structure of different *YDA* mutants.
- Identification of interacting proteins of YDA in vascular tissue.
- Search for conserved motifs of promoters of vascular-expressed genes in vascular and non-vascular plants, including *YDA*.

MATERIALS AND METHODS

4 MATERIALS AND METHODS

4.1 Plant material

YDA mutant seeds were obtained from the ABRC stock center. *YDA* mutant seeds and WT plants (Ler and Col-0) were stratified at 4°C for 2 days and then sown on soil/peat moss/agrolite (4:4:2). Seed germination and growth were carried out in growth chambers (CONVIRON) with the following conditions: relative humidity 70%, temperature 22°C, and 16h light/8h dark photoperiod (long day conditions).

YDA alleles with the following accession numbers: CS6392 (*yda-1;* Lukowitz *et al.*, 2004), CS6392 (*emb71,* Meinke *et al.,* 2009) and Y295 (Bergmann *et al.,* 2004; Lukowitz *et al.*, 2004) were obtained from the ABRC stock center.

Nicotiana benthamiana and *Cucurbita maxima* were grown for expression of recombinant YDA. *N. benthamiana* plants were grown in peat pellets as described previously (Leuzinger *et al.*, 2013) in growth chamber with 70% relative humidity, 22°C and long-day conditions.

Pumpkin plants were grown on soil under long-day conditions and at 28°C in a growth chamber (Yoo *et al.*, 2004, Taoka *et al.*, 2009, Ham *et al.*, 2009, Ma *et al.*, 2010, Notaguchi *et al.*, 2012).

4.2 Arabidopsis DNA extraction

DNA extraction for WT and transformed plants was performed as follows: The tissue was frozen and homogenized using CTAB extraction buffer: 100 Mm Tris-HCl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, 2% CTAB, 1% PVP and heating at 65°C for 30 minutes. Then 1 volume of phenol: chloroform: isoamyl alcohol was added to the homogenized and centrifuged. Supernatant was collected and supplemented with CTAB.

The mixture was heated at 65°C during 15 minutes and then chloroform: isoamyl alcohol (24:1) was used to separate the phases. Supernatant was collected and precipitated with isopropyl alcohol. Samples were centrifuged, washed 3 times with ethanol at 70%, dried and resuspended in 30 μl of Milli-Q water. DNA integrity was verified on agarose gel 1% stained with Ethidium bromide and quantified by spectrophotometry with NanoDrop -2000 (Thermo Scientific, Waltham MA).

4.3   Molecular Biology Procedures

Promoter sequences from YDA, *IRX3*, *SUC2* and *APL* were amplified from genomic DNA. 1.5 kb upstream of the *YDA* open reading frame was used as promoter and amplified using TAKARA polymerase (Table S1). The length of *SUC2* and *IRX3* promoters were taken from Uchida *et al.* (2012) while the APL promoter length was taken from Bonke *et al.* (2003). The fragments were ligated in pCR8/GW/TOPO cloning vector (Invitrogen, La Jolla CA) at 22°C overnight (Table S2). *E. coli* DH5α was transformed by heat shock and grown on spectinomycin at a final concentration of 100 μg/ml.

Restriction enzymes were used to release the fragments and check their orientation on vector (Table S6). Positive clones were subcloned in the plant expression vector pBGWFS7 (Plant Genetic Systems, Ghent, Belgium) using LR clonase II essentially as recommended by the manufacturer (Invitrogen) (Table S3).

An amplicon of approximately 5 Kb of length corresponding to the full genomic locus of YDA (pYDA:YDA) was cloned into pCR8/GW/TOPO cloning vector using *E. coli* DH5α. Positive clones were verified as mentioned. pB7FWG,0 vector was used for subcloning, and generating a GFP fusion.

The complete *YDA* cDNA was obtained from the ABRC stock center (www.arabidopsis.org) with the accession number CD3-638. A fragment of 2.6 kb fragment was cloned into pCR8/GW/TOPO and subcloned in the plant expression vector pEarley gate 103 (Earley *et al.,*

2006) harboring GFP and controlled by the 35S promoter (Figure 5). Primers and conditions used for amplification of these regions are shown in Table S4.
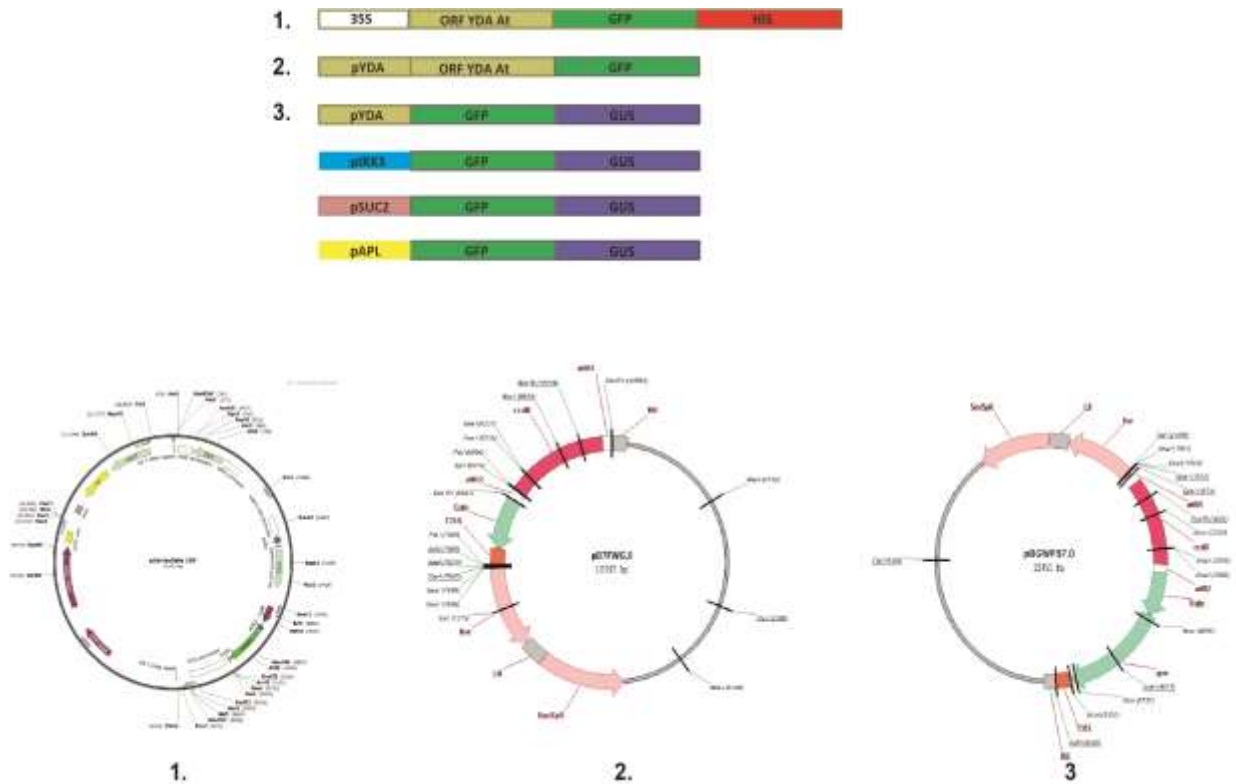
Figure 5. Scheme of constructs obtained for this work. The YDA ORF was cloned in pEARLEY GATE 103 vector, Full length YDA was cloned into pB7WG,0. YDA, APL, IRX3 and SUC2 promoters were cloned in pB7GWFS7.

4.4 Plant transformation

The constructs for plant expression were introduced into Arabidopsis thaliana WT ecotype Col-0 by the floral dip method using *Agrobacterium tumefaciens* C58C1 (Clough and Bent 1998). Seeds were collected and sown in soil. Plants were sprayed 3 times once a week during three weeks, with ammonium glufosinate (300 μM) for selecting transgenic plants.

Genomic DNA was used to detect the *GFP* gene in plants harboring *YDA* ORF, and *bar* in plants harboring the *YDA* promoter construct by end point PCR. The size of GFP correspond to 720 bp and BAR 500 bp. Sequences and amplification cycle are detailed in the (Table S5).

4.5   GUS staining for promoter analysis

GUS histochemical assay was performed essentially as described in Weigel and Glazebrook (2002). Plant tissues (leaves, stem, flowers, siliques) were cut and stained with GUS solution 0.5 mM (1mM X-Gluc (Thermo Scientific) dissolved in DMSO, 100 mM phosphate Buffer pH 7.0, 0.1% Triton X-100, 10Mm EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide) and infiltrated by vacuum. Tissue was incubated at 37°C during 16 hours, and destained with ethanol:acetic acid and acetone:methanol (3:1 in both cases). Plant tissues were mounted and observed under a three-ocular Nikon SMZ745T dissecting microscope (Nikon México). To observe vasculature cells, inflorescence stems were embedded in 1% agarose, frozen in liquid nitrogen and cut with a Microm microtome (Thermo Scientific). The sections were visualized by Nikon Optiphot-2 microscope. Images were taken with a Nikon camera using NIS-Element software.

4.6   Confocal Microscopy

Plants positive for GFP by PCR were cut and fixed in agarose 1% and deposited on blocks. The agarose was frozen in liquid nitrogen. Slices of transversal sections of inflorescence stems were obtained and visualized in a TCS-SP5/MO-TANDEM Leica confocal microscope (20X, 40X and 63X objectives: HCxPLAPO -blue IMM UV 0.7 NA; Leica, Germany) with a laser Tisaph 200M 2X (resolution 1024 x 1024 pixeles). The wavelength used ranged between 515 nm/ 580 nm for excitation and 525-550 nm/600-680 nm for GFP and auto-florescence detection. Images were collected and processed using Leica Las AF.

4.7 Histological Analysis of YDA mutants

Stem inflorescences of 1 cm tall were taken from the *YDA* mutants and WT *Arabidopsis thaliana* plants (Altamura *et al*., 2001). The tissue was fixed in FAA (formaldehyde 3.7%, acetic acid 5%, ethanol 50%), and embedded in wax to obtain transverse sections (5-10 μm thick).

The slides were incubated at 42°C overnight, after which wax was removed with Citrisolv (Thermo Scientific) during 10 minutes and stained as follow:

The o-toluidine blue polychromatic staining was used to observe the structure of vascular tissue. The reaction was carried out as follows: 0.05 % toluidine blue in 0.1 M phosphate buffer at pH 6.8 during 1 minute (O'Brien *et al.,* 1964, Mähönen *et al.,* 2000, Altamura *et al.,* 2001, Bonke *et al,* 2003, Mähönen 2006, Muñiz *et al.,* 2008, Yamaguchi *et al.,* 2011, Bryan *et al.,* 2012).

For phloroglucinol-HCl staining, the reagent was prepared as follows: phloroglucinol 2% in ethanol mixed with concentrated HCl in a 2:1 ratio, and tissue sections stained for up to 1 hour and observed under an optical microscope. The lignified tissue was stained red (García and Latgé 1987, Ni *et al*., 1994, Franke *et al*., 2000, Elfstrand *et al*., 2001, Bonke *et al*., 2003, Bryan *et al*., 2012). Images were obtained with a Nikon Optiphot 2 microscope and camera using NIS-Element software.

4.8 YDA expression levels under hormones treatments

Arabidopsis WT plants were used to analyze the *YDA* expression pattern under different hormonal treatments. Arabidopsis seeds disinfection was made washing wih 5%sodium hypochlorite during 10 minutes, then ethanol 70% during 5 minutes followed by 5 washes with sterile water in a sterile hood. Seeds were spread into MS medium (Murashige & Skoog, 1962 supplemented with inositol (100 mg/l), thiamine (1 mg/l), potassium phosphate (180 mg/l), 2-4 dichlorophenoxyacetic acid (2 mg/l) sucrose 1%(15 g/l), pH 5.8). Adult plants germinated in peat pellets and left there until their bolting.

For this assay, we used the main plant hormones: ABA (abscisic acid), gibberellic acid (gibberellin), indole-3-acetic acid (auxin), kinetin (cytokinin), and brassinolide (brassinosteroid). The hormone concentrations were adopted from Weigel and Glazebrook (2002) (Table S7).

Two weeks after germination, seedlings and 6-week-old adult plants were used for the assays. The plants were exposed to growth regulators in MS during 16 h and using medium as mock. Two pools of 6 plants were used for each treatment and tissue was collected and used for RNA extraction.

4.9 RNA extraction

Total RNA was obtained from complete seedlings and from different organs of treated plants (leaves, stem, apex, roots). Extraction was done using Direct-zol RNA miniprep kit (Zymo Research, Irvine CA) according to the manufacturer´s specifications. Samples were treated with DNAse (Zymo Research) and quantified with a Nanodrop 2000 (Thermo Scientific).

4.10 Quantification of YDA expression levels

Concentrations of RNA sample were normalized to 50 ng/ml and the reaction mix was done using Kapa Sybr-Fast qRT-PCR, following the manufacturer's instructions. A Step One equipment (Applied Biosystems, Thermo Scientific) was used. As reference gene in Arabidopsis we used polyubiquitin 10 (UBQ10; accession no. At4g05320.2). Relative quantification was calculated by the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001). Primers and amplification conditions are specified in Table S8.

4.11 Expression analysis of cucumber YDA orthologs

In silico analysis of *Arabidopsis thaliana* YDA (At1g63700) showed that it has two homologs in Cucumber (*Cucumis sativus*) with the following accession numbers: *Cucsa107950* and

Cucsa129470. RNA was obtained from vascular bundles and leaves as mentioned above. The expression pattern of both genes in vascular tissue was determined by qRT-PCR using actin 8 from Arabidopsis (ACT8; accession no. At1g49240) (Table S9) as reference gene and calculating the relative expression by the $\Delta\Delta$CT method.

4.12 YDA Protein expression assays

4.12.1 Cloning in ZYMV vector

Fragments corresponding to the N-terminal domain, MAPK domain and C-terminal domain were amplified with the blunt-end polymerase Phire Phusion (Thermo Scientific) (Table S10). Primers with *Sph*I and *Kpn*I restriction sites were used to obtain each fragment and cloned into the pZero Blunt TOPO vector (Invitrogen) (Table S11). Fragments were released by digestion with these enzymes, and purified with the Zymo Clean Gel recovery kit. ZYMV vector was linearized and used to ligate the purified fragments (Table S12). *E. coli* DH5α was transformed by heat shock and grown in plates containing carbenicilin 100 μg/μl.

4.12.2 Biolistic transformation

Viral vector containing YDA fragments were used for pumpkin inoculation by microprojectile bombardment following the method described by Lin *et al*. (2007). Gold particles of 1 mm were covered with the ZYMV vector using spermidine 50 mM and CaCl$_2$ 1M to stick the DNA (~1mg). The mixture was washed and resuspended in ethanol 100%. 15 μl were loaded onto the macrocarriers. Shots were done on the cotyledon of pumpkin seedlings that had one true leaf at 100 psi using a biolistic helium gun (PDS-1000; Bio-Rad; Hercules CA).

4.12.3 Protein extraction and purification from pumpkin

Leaves that showed ZYMV symptoms were collected 8 days after biolistic delivery of the recombinant virus. Tissue was disrupted with liquid nitrogen and adding His extraction Buffer: 10 mM Tris-HCl pH 8, 50 mM NaCl, 10% glycerol, and 0.1% TritonX-100. The extract was sonicated and filtered to remove cell debris using 0.45 μm and 0.22 μm pore filters. The filtrate was used for purifying the desired protein by affinity using a His trap FF crude column (1ml GE) and the elution buffer containing 500 mM imidazole. The fractions were collected and examined by Western blot (Table S14) using a monoclonal c-Myc antibody (Santa Cruz Biotechnology). Positive fractions had a second round of purification by immunoprecipitation using the Pierce c-Myc tag IP/Co-IP kit (Thermo Scientific) according the manufacturer's instructions and then verified by Western Blot.

4.13 Cloning of YDA in pTrcHis2 TOPO

The 2.6 kb fragment corresponding to the *YDA* ORF was also used for protein expression assays in bacteria. This region was amplified using the ORF primers previously mentioned and cloned directly into pTrcHis 2 TOPO vector (Invitrogen) (Table S13). The DH5α strain of *E. coli* was transformed as has been described, and verified by sequencing.

4.13.1 Expression assays in E. coli

E. coli BL21 and Rosetta 2 strains were transformed with the positive clone and used for expression assays. TB (Table S16) medium supplemented with 0.2% of glucose and 100μg/μl carbenicilin. 35 μg/μl chloramphenicol was also added only in the case of Rosetta 2 induction was done using IPTG at 0.5 mM during 2 hours. To determine the adequate induction time assays at different time lapse were first performed.

4.13.2 Protein extraction and purification from bacteria

Cells were collected and disrupted using the aforementioned His protein extraction buffer, adding lysozyme 10mg/ml during 15 minutes at room temperature. Freeze/thaw cycles were carried out using dry ice. Sonication was performed and then soluble and insoluble fractions were separated by centrifugation.

Soluble and insoluble fractions were analyzed by Western Blot (Table S14). YDA soluble fraction was precipitated by 40% of ammonium sulfate during 1 hour at 4°C (EnCorbio, Gainesville FLA; http://www.encorbio.com/protocols/AM-SO4.htm) and centrifuged. The pellet was solubilized, dialyzed in His extraction buffer and filtered.

Purification was made using a FPLC (Äkta Pure, GE, Pittsburgh PA) for affinity and ionic exchange purification.

4.13.3   Agroinfiltration of N. benthamiana

The ORF YDA construct was used for plant agroinfiltration. *Agrobacterium tumefaciens* C58C1 was grown at 28 °C in LB medium (Table S17) containing Spectinomycin 100 μg/μl, Tetracyclin 5 μg/μl and Rifampycin 50 μg/μl. Bacteria was collected and resuspended in infiltration media: MES, MgCl$_2$ and 100 mM acetosyringone. 6 week-old plants were infiltrated with the bacteria solution, using vacuum (Leuzinger *et al*., 2013). After 5 days leaves were harvested, visualized using a UV lamp and then used for protein extraction following the same protocol used for pumpkin. The extract was analyzed by Western blot (Table S14) using polyclonal GFP antibody (Santa Cruz Biotechnology).

4.1   *In silico* analysis of YDA and potentially vascular-expressed promoters

The 1500 bp upstream sequences corresponding to promoters of the homologs of genes expressed in vasculature in Arabidopsis (Ruiz-Medrano *et al*., 2011) from vascular, non-vascular plants and chlorophytes were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Motif analysis for these promoters was performed

using the Multiple EM for Motif Elicitation method (MEME; http://meme.nbcr.net/meme/; Bailey and Elkan, 1994). Common motifs found in the promoters were represented schematically using the Weblogo program (http://weblogo.threeplusone.com/create.cgi; Crooks *et al*., 2004).

4.2   Phylogenetic Analysis

FT, APL, OPS and YDA full protein sequences from Arabidopsis and CmPP16 from pumpkin were retrieved from TAIR (www.arabidopsis.org) and Phytozome). BLAST analysis with other proteome plants was done in the Phytozome database (www.phytozome.net). Protein alignment was performed by CLUSTAL X2 (Larkin *et al*., 2007) and edited manually in Seaview4 (Gouy *et al*., 2010).

Maximum-Likelihood and Neighbor-Joining phylogenies were used to obtain the trees in MEGA5 (Tamura *et al*., 2011) with 1000 bootstraps.

RESULTS

5 RESULTS

5.1 Analysis of *YDA* promoter expression

5.1.1 Plant promoter analysis

YDA promoter clones were verified in pCRGW8 TOPO and in the plant expression vector PBGWFS7. According to the restriction analysis in pCRGW8 TOPO with *Eco*RI and *Hinc*II (Figure 6), two clones contain the fragment, in the plus orientation, and were then used for LR
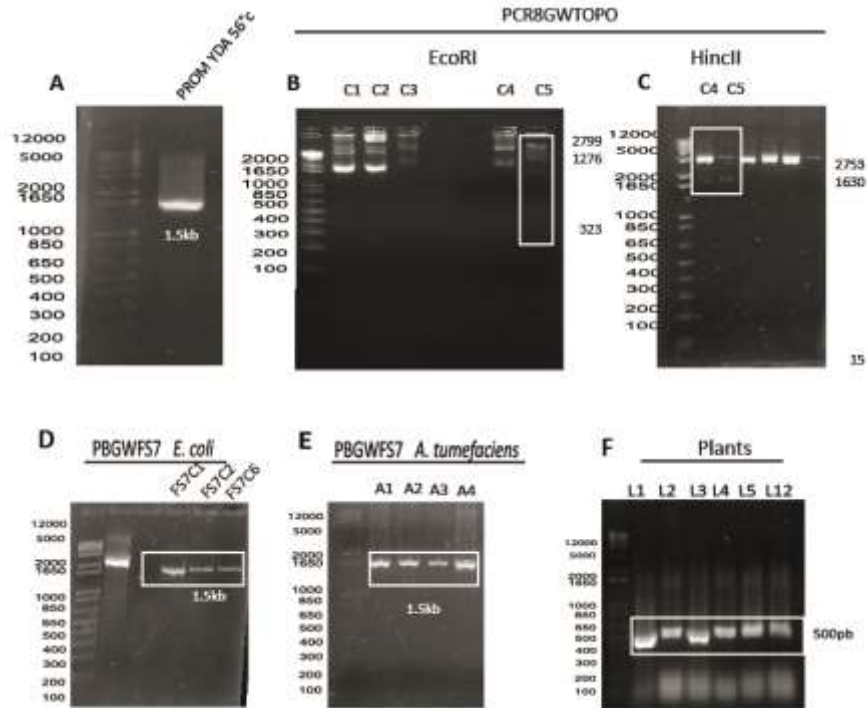
Figure 6. *YDA* **Promoter cloning process**. A) YDA PCR at 56°C B, C) Presence of the insert and orientation were verified in PCR8GWTOPO using EcoRI and HincII . D-E) Presence of the promoter fragment was confirmed by PCR in the PBGWFS7 in both E. coli and *A. tumefaciens*. F) Indepent lines were selected and analyzed by PCR to confirm their transformation amplifying the BAR gene

clonase reaction to subclone in the plant vector. Three clones were positive by PCR containing the 1.5 kb insert in the pBGWFS7 vector and were subsequently transformed into the *Agrobacterium tumefaciens* C58C1 strain. Likewise the resulting Agrobacterium transformant colonies were verified by PCR with the *YDA* promoter-specific primers, of which 4 were positive.

One clone was selected for cryopreservation and it was also used to transform Arabidopsis plants by floral dip (Clough and Bent 2008).

The seeds from transformed plants were collected and selected in soil using ammonium glufosinate. The surviving plants were treated as independent lines and transformation was confirmed PCR for the *bar* gene.

Six herbicide-resistant lines were obtained, and GUS analysis was performed in T2 and T3 plants. Promoter expression was observed in different tissues, particularly in veins of rosette leaves. The pattern varies in cauline leaves, being expressed in minor veins but not in the midvein. In mature flowers the expression is mainly localized to carpel, and GUS stain occurred in the base of the silique and up to the apical region of the silique, but only in the vasculature. Axillary buds and growing tissues displayed strong GUS staining, while in the inflorescence stem signal was detected in the vascular bundles. In transverse sections of stems, expression was restricted to phloem and xylem but was absent in cambium (Figure 7).
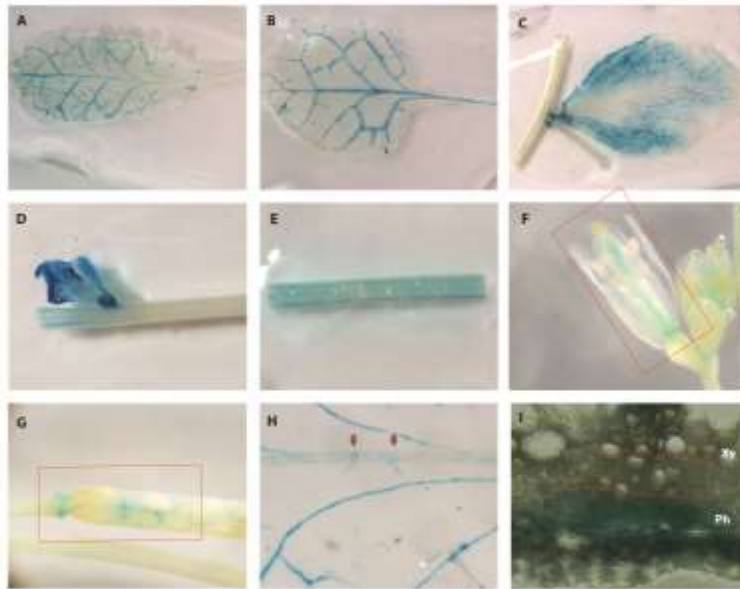
Figure 7. *YDA* promoter expression in adult plants. A) Rosette leaf 7. B)Rosette leaf 2. C) Cauline leaf. D) Stem and young leaf. E) Inflorescence stem. F) Mature flowers. G) Silique. H) Trichomes on leaf surface. I) Hand-cut cross section of inflorescene stem. Red rectangles show carpel in image F and vasculature of siliques in G. Red arrows show trichomes on leaf. Ph: phloem, Xy: xylem

5.1 Cloning of *YODA* ORF

The *YDA* ORF (At1g63700) was cloned and confirmed by restriction analysis in pCR8GWTOPO. The restriction pattern with *Xba*I showed four positive clones (Figure 8B). These were used for the LR clonase reaction to subclone into the pEarley gate 103 vector. Restriction analysis of pEarley gate 103 clones showed the expected patterns in three clones from *E. coli* (Figure 8C). Positive clones were transformed into *A. tumefaciens* and then verified by PCR using the specific primers to amplify *YDA ORF*. One clone was selected and used for plant transformation
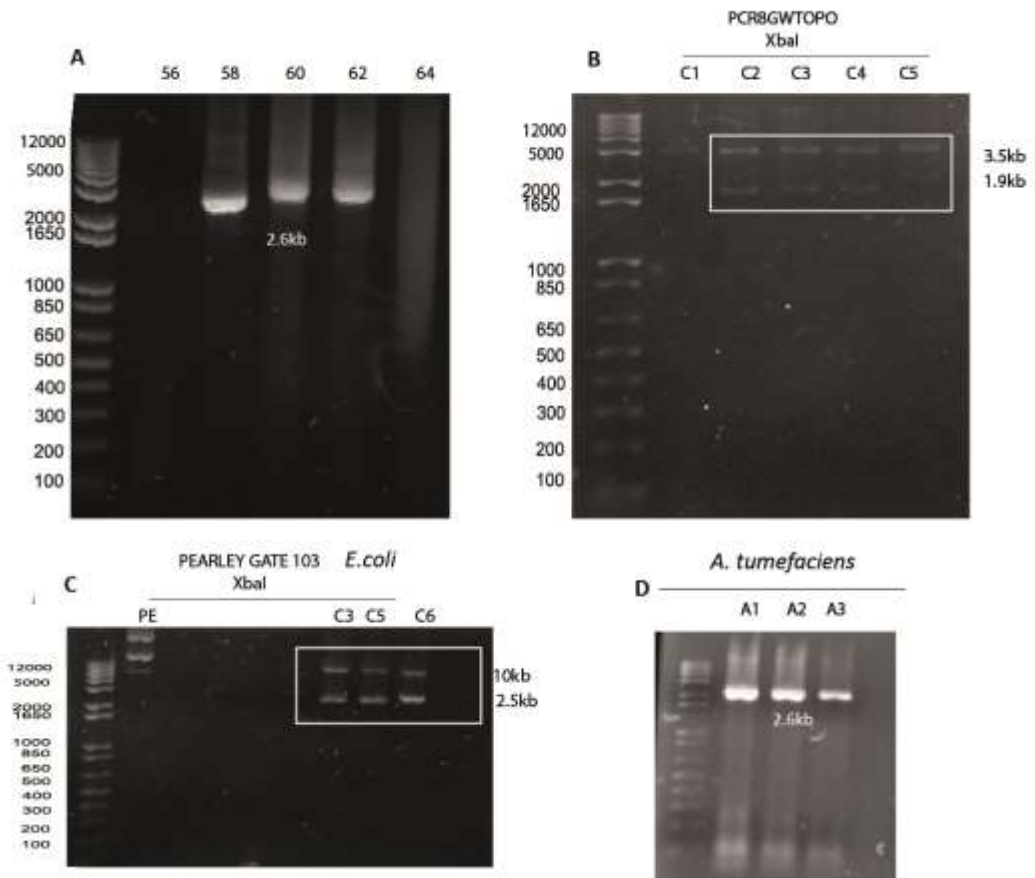
Figure 8. Cloning process of YDA ORF. A) PCR gradient showing 3 temperatures of amplification. B) Orientation of fragment by restriction enzyme XbaI in PC8GWTOPO C) Fragment verification into the pEarley gate 103 vector by *Xba*I digest in *E. coli.* D) *A. tumefaciens* clone verification by PCR with specific primers

Transformed plants were selected with herbicide in soil. Five lines were confirmed by PCR with GFP but only 4 lines survived. Compared with youngest WT, plant differences in growth were observed between each line, although it is likely due to herbicide selection (Figure 9).
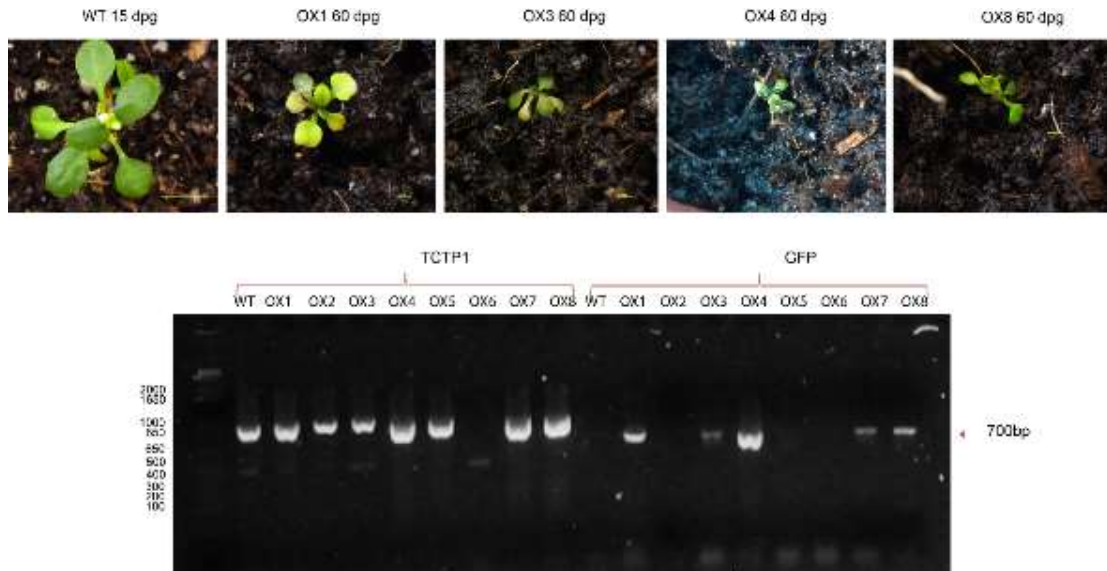
Figure 9. Selection of *YDA* ORF plants and verification by amplification of GFP. Comparison of a WT plant with selected T1 plants. B) PCR showing amplification of TCTP as an endogenous control and amplification of GFP in 5 lines.

Four T2 lines were harvested and sown in order to analyze GFP fluorescence, and thus YDA accumulation, by confocal microscopy. Inflorescence stems in longitudinal and cross sections show protein localization mainly in vasculature, including structures that resemble sieve plates. GFP-associated fluorescence was also observed in vascular bundles similar to that in plants harboring the *YDA* promoter fused to GFP and GUS. GFP possibly also localized in sieve elements (Figure 10).
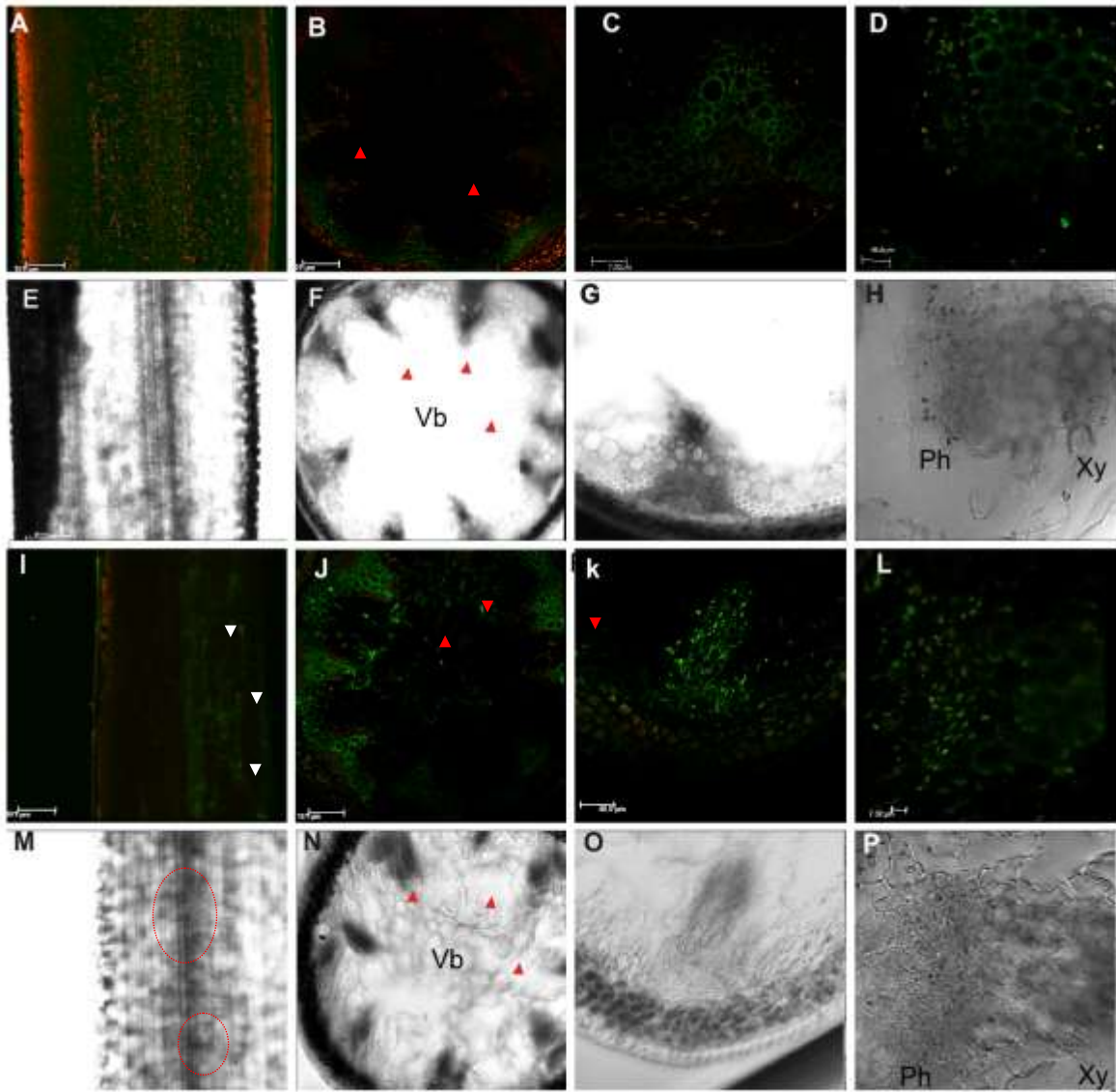
Figure 10. Confocal microscopy analysis of *35S::YDA ORF:GFP* plants. A-H WT inflorescence stem, longitudinal (A and E) and cross sections (B- D and F-H). I- P, YDA-GFP inflorescence stem, longitudinal (I and M) and cross sections (J-L and N-P). Red triangles: Vascular bundles (Vb), White triangles: Sieve plates. Ph: phloem, Xy: Xylem.

5.2 Cloning of *YDA* genomic locus and *SUC2*, *IRX3* and *APL* promoters

Arabidopsis full-length genomic locus was amplified using the promoter forward primer and the *YDA* ORF reverse primer. Restriction analysis with *Xba*I showed the plus orientation in the pCRGW8TOPO vector. The clone 3 was selected for subcloning. The positive clone was subcloned in the pB7FWG,0 vector and verified in *E. coli* by PCR with primers that amplify a 200 bp *YDA* fragment corresponding to the carboxy end of the protein (COOH YDA primers; Figure 11). This clone was transformed *into A. tumefaciens* and the insert verification was made by PCR like it had been done for *E. coli*
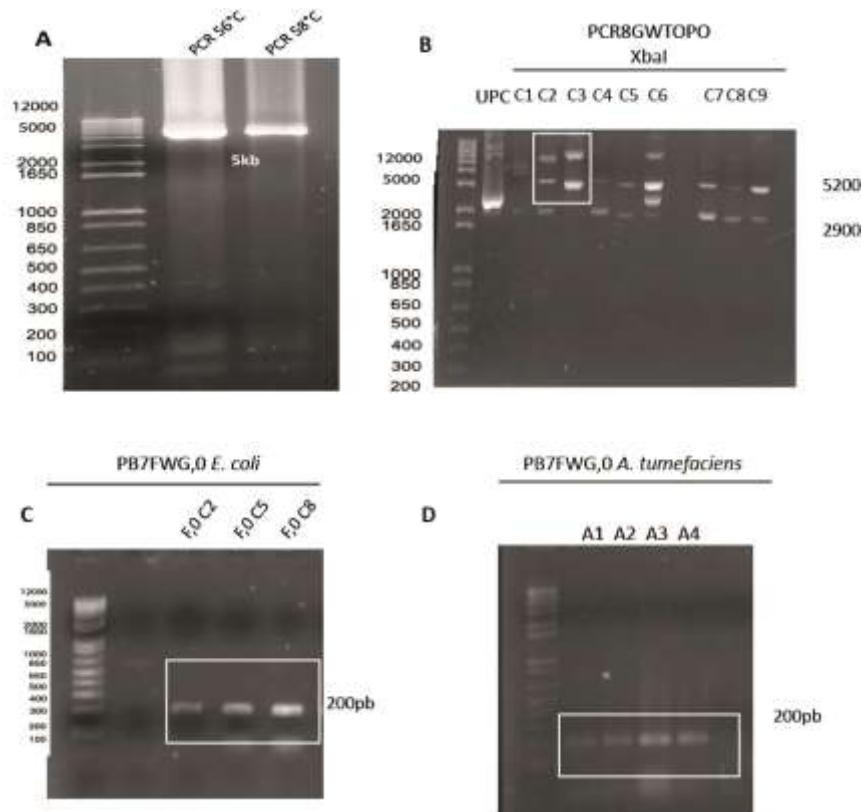
Figure 11. PCR products of the *YDA* full length genomic locus. A) PCR products obtained at 56°C and 58°C showing the expected 5 kb product. B) Restriction analysis with XbaI in pCRGW8TOPO. C, D) PCR using COOH YDA primers to amplify 200 bp in binary vector in *E. coli* and *A. tumefaciens*

In order to visualize defects in specific tissues in *YDA* mutants, if any, *SUC2*, *IRX3* and *APL* gene promoters were fused to GFP. These were first amplified and cloned in pCRGW8TOPO. *SUC2*, *IRX3* have been used as reporter genes of CC and protoxylem, respectively (Imlau *et al.,* 1999, Gardiner *et al.,* 2003, Uchida *et al.,* 2012). APL promoter was used due to its function being more related to SE and CC formation (Bonke *et al.,* 2003). Restriction analysis by *Eco*RV of these three promoter constructs generated the expected band pattern for the right orientation. The positive clones were subcloned in pB7GWFS7 and verified by PCR with the corresponding primers (Figure 12).
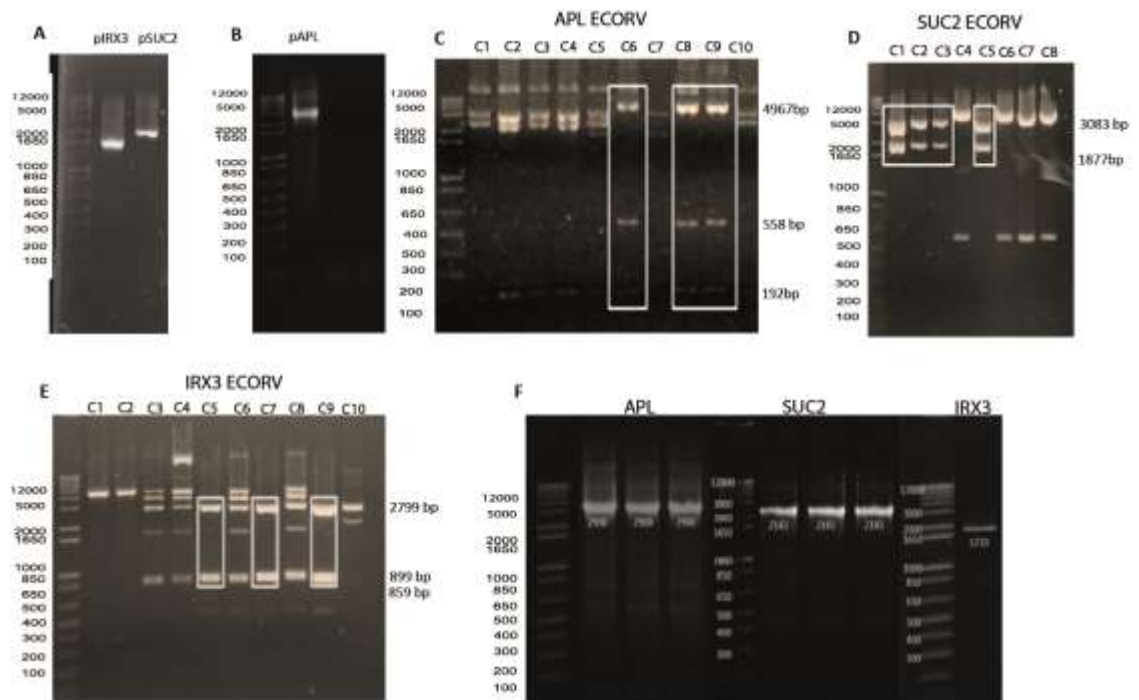
Figure 12. Cloning process of the three promoter *APL, IRX3* and *SUC2*. A, B), Amplification of these promoters. C) Restriction analysis of pAPL clones with EcoRV. D) Restriction analysis of pSuc2 clones with EcoRV. E) Restriction analysis of pIRX3 clones with EcoRV. F) PCR of three promoters in pB7GWFS7 in *E. coli*.

5.3 Phenotype analysis of vascular tissue in *YDA* mutants

5.3.1 Histological Analysis

YDA mutants presented growth delay relative to WT plants of the corresponding ecotype (Ler; Figure 13). The surviving percentage of mutants was between 10 to 15% for *yda-1* due to the role of YDA in suspensor elongation. The most severe phenotype was observed in *emb71* mutants, which displayed very small rosettes; only 5 to 8 % of these plants survived past the bolting stage. Figure 10 shows the phenotype of *emb71* adult plants compared with WT plants. The former are quite small, while the WT that germinated at the same time have almost finished their life cycle. A map based on Lukowitz *et al.* (2004) is also shown in Figure 13; the *yda-1* mutant harbors a premature stop codon substituting a glutamic acid in the ninth exon, producing a non-functional protein. In the case of *emb71* there is no information about the mutation site or the nature of the mutation (Meinke *et al.*, 2009).

Heterozygous plants were used to analyze the inflorescence stem, 8 different plants for *yda-1* and 5 for *emb71* using as bolting parameter a length of 1 cm of the stem (Altamura *et al.*, 2001).
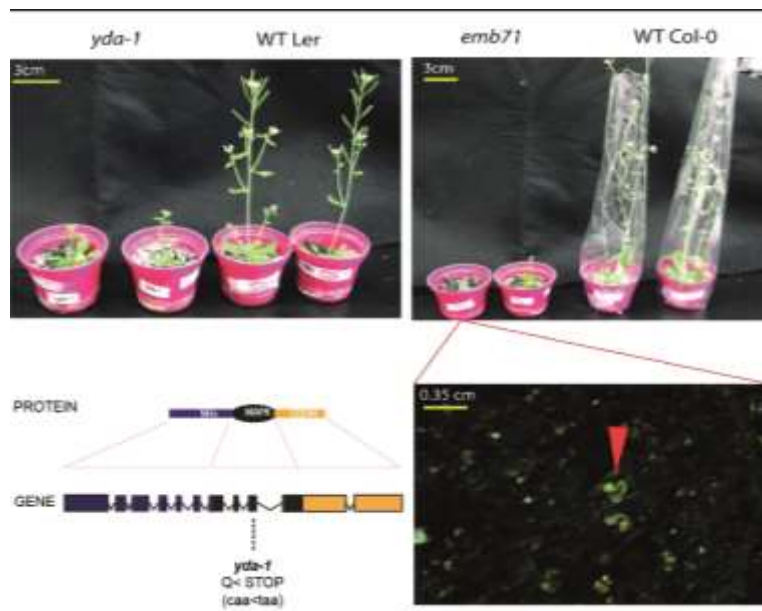
Figure 13. Phenotype of *yda-1* and *emb71* heterozygous plants. Upper panel: yda-1 and emb71 plants compared with WT Ler and WT Col-0 at 47 days after germination, respectively. Lower panel: Left, scheme of mutation site in yda-1 plants; right, close up of emb71 6 weeks post germination. Bar 0.35 cm

In *emb71* plants, the stem shows alteration in diameter and shape with respect of the WT Col-0 plants (Figure 14B, D). Arrangement of vascular bundles in the stem is irregular, and appears to have a decreased number of bundles in the mutant compared to WT. Upon a closer inspection of the vasculature, a decreased number of cambium and phloem cells was observed. Phloroglucinol staining shows lignified tissue in red, while o-toluidine produces a polychromatic staining, displaying different blue tones in different cells.
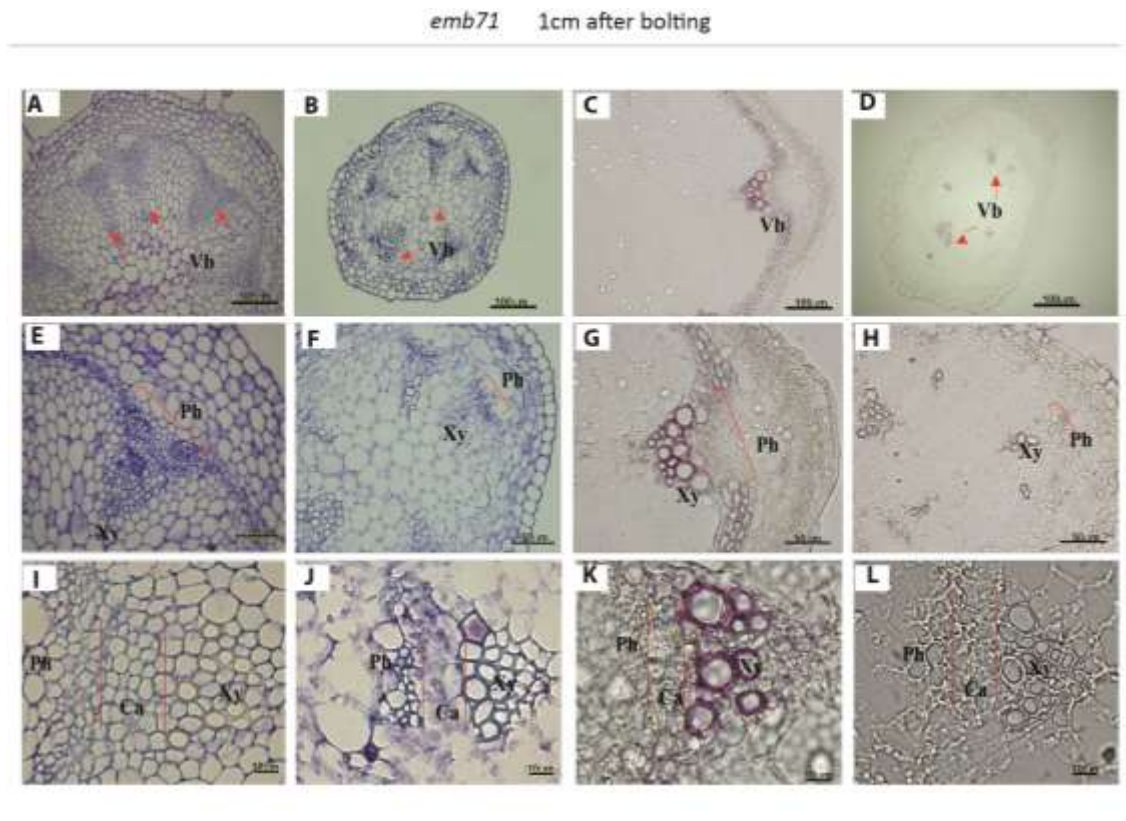
Figure 14. Phenotype of vascular tissue in the inflorescence stem of representative *emb71.* O- toluidine staining in WT – Col-0 (A, E, I) and *emb71* mutant (B, F, J). Phloroglucinol staining in WT-Col-0 (C, G, K) and *emb71* plants (D, H, L). Mutations in YDA result in a smaller overall size of the plant, as well as a decrease in stem size and abnormal arrangement of vascular bundles. Specifically, in vascular tissue it results in a compressed vascular bundle. Vb: Vascular bundle, Ph: phloem, Ca: cambium, Xy: Xylem. Bars,100 μm, 50 μm and 10 μm when indicated.

Similar defects were found in *yda-1*. In Figure 15C a deformed stem with smaller girth is observed in a cross section relative to WT Ler. Vascular bundles have asymmetric shape and smaller size when compared with the normal phenotype of the vascular bundle. As has been observed in *emb71*, the vasculature cells appear to not completely develop, or with decreased number of cells, specifically in phloem and cambium cells, generating a short and flat vascular bundle phenotype (Figure 15).
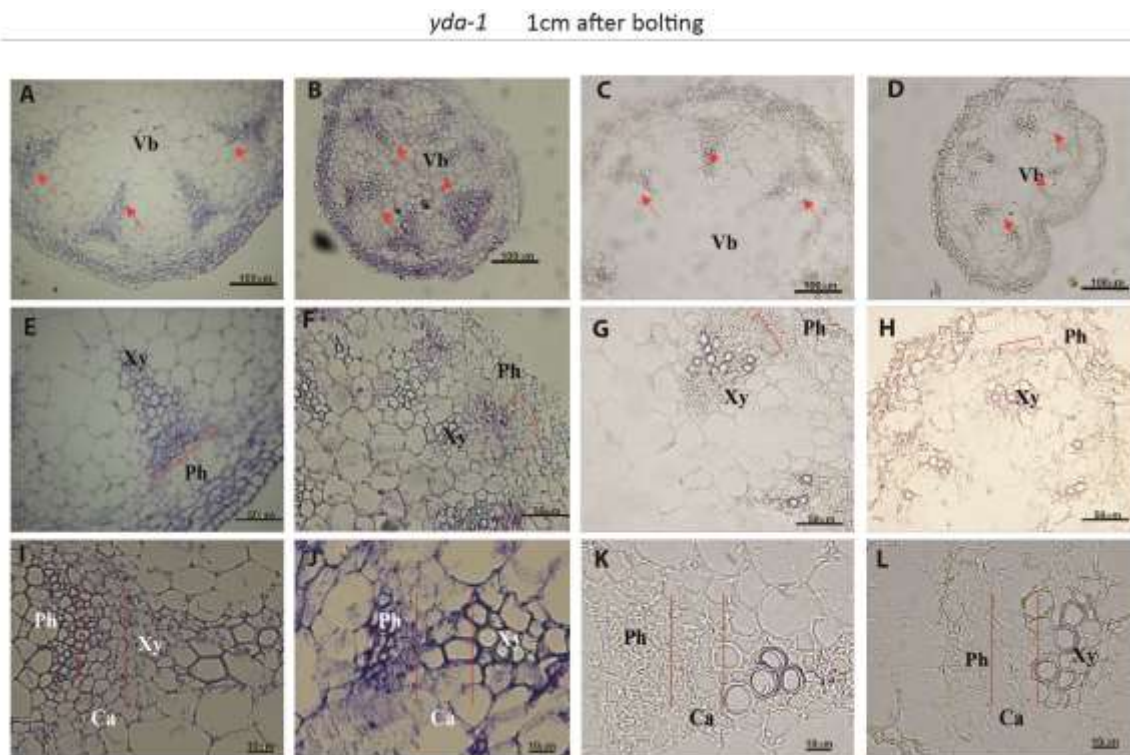
Figure 15. Phenotype of vascular tissue in the inflorescence stem of representative *yda-1.* O- toluidine staining in WT – Col-0 (A, E, I) and yda-1 mutant (B, F, J). Phloroglucinol staining in WT-Col-0 (C, G, K) and yda-1 plants (D, H, L). Mutation in YDA gene produces small stem and unpaired pattern of vascular bundles resulting in flattening of vasculature in stem inflorescence. Vb: Vascular bundle, Ph: phloem, Ca: cambium, Xy: Xylem. Bar of 100 μm, 50 μm and 10 μm.

5.4 Analysis of *YDA* transcript levels in hormone-treated plants

It has been reported that the *YDA* gene does not respond to the main plant growth regulators, at least at the transcriptional level (Lukowitz *et al*., 2004); however this was only tested in one developmental stage. Moreover, the presence Y*DA* mRNA in different tissues and organs suggested to us that this may not be the case, and that the *YDA* gene could respond in an as yet unspecified manner to growth regulators. Supporting this, recent work points to a possible regulation of *YDA* by at least some plant growth regulators (Kim *et al*., 2012; Smékalová *et al*., 2014).
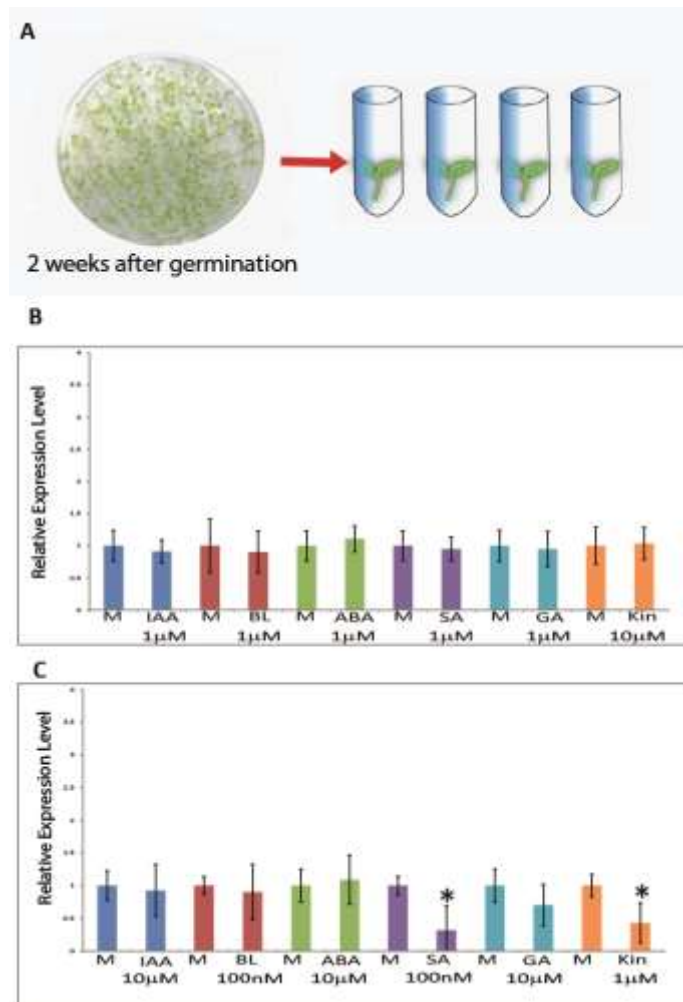
Figure 16. *YDA* is transcriptionally repressed by cytokinin and SA during seedling stage. A) Seedlings treated after 2 weeks growing in MS. B, C) Graphics showing relative expression under hormone treatment. Asterisks show repression of the YDA transcript at 100 nM and 1 μM of kinetin.

We decided to test *YDA* expression levels at different developmental stages in response to the main plant growth regulators, auxin (IAA), abscisic acid (ABA), cytokinin, brassinolide (BL) and salicylic acid (SA) at two different concentrations. As previously reported, the *YDA* transcript levels remained unchanged at the highest concentrations tested; however, we could observe downregulation of *YDA* at low concentrations of cytokinin and salicylic acid (Figure 16), while *YDA* response to the other treatments again remained unaltered.
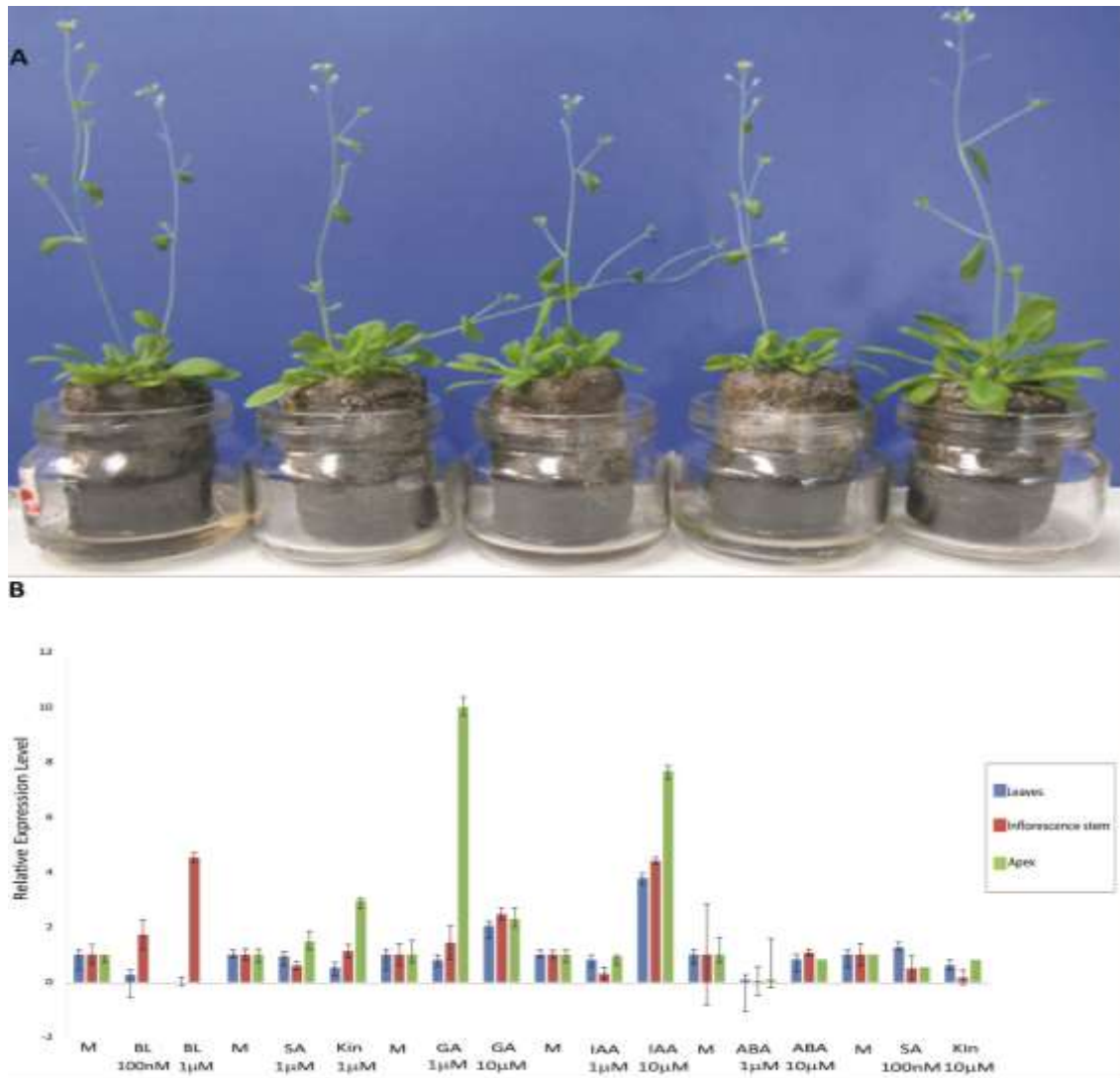
Figure 17. *YDA* expression levels under growth regulators in different tissues. A) Image of plants grown in peat pellets and treated with the corresponding growth regulators in BNS 1X medium. B) Relative expression in leaves, inflorescence stem and apex.

To determine whether *YDA* transcription is affected during adult stages and in different tissues, we used pools of leaves, apices and stem tissues. In contrast with seedlings, in adult plants we could observe some level of variation in *YDA* expression. All the assays had problems due to variations in expression of the normalizer gene, UBQ10. This was selected for these assays since it is a common normalizer gene, and broadly used. Indeed, it also showed constant transcript levels in the seedling stage. As IAA, ABA and BL exposure promote upregulation of UBQ10. we consider convenient to analyze other genes that do not change their expression under these conditions such as the subunit of the protein phosphatase PP2A (At3g25800) which is in the Top 100 of genes used for root biotic stress and RIKEN hormone series and the elongation factor EF-1α (At5g60390) also used in the RIKEN hormone series (Czechowski *et al.,* 2005). We should mention that although we had problems in our analysis, we could observe, based on the CT raw data from *YDA*, that it is in fact induced three-fold under 1 μM BL in stems, suggesting that brassinosteroids could regulate *YDA* at the transcriptional level, in addition to the post-transcriptional regulation by BIN2 (Kim *et al*., 2012). *YDA* is repressed by 1 μM SA in leaves, suggesting a role for YDA in the biotic stress response. We also observed an approximately twofold induction of *YDA* levels with a 10 μM IAA treatment in leaves and twofold induction under 10 μM GA treatment, again suggesting that these hormones could regulate *YDA* transcript levels in these tissues (Figure 17).

5.5 YDA protein expression analysis

5.5.1 *In silico* analysis of YDA for heterologous expression

ZYMV (*Zucchini yellow mosaic virus*) is a virus-based vector which was used for expression and purification of YDA in pumpkin in order to provide protein for coimmunoprecipitation assays. This in turn would be used to determine interaction partners of YDA. This vector has been used successfully for the analysis of FT phloem transport in cucurbits (Lin *et al*., 2007). Before cloning in the ZYMV vector, *in silico* analysis was performed to determine whether there are Arabidopsis YDA homologs in cucurbits that are sufficiently related; if this were the case, it could be expected that interacting partners are also present in these species. Thus, Arabidopsis YDA could be expressed in such species to purify it and use it for immunoprecipitation assays. The system to express the protein was pumpkin, as already mentioned, although its genome is not completely sequenced. Phylogenetic analysis was performed using the YDA cucumber and watermelon orthologs as well as other reported orthologs from *Oryza sativa* and *Populus trichocarpa*, which were compared with other members of Group A1 MAPKKK to which YDA belongs (MAPK Group 2002). The MAPK domain was used to perform a phylogenetic reconstruction (Figure 18).
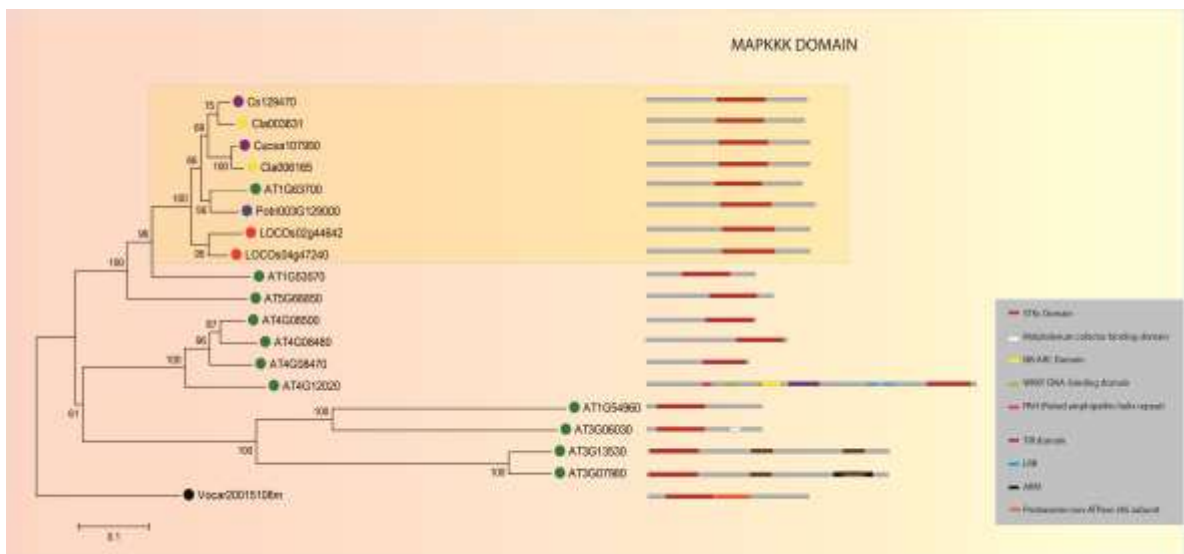
Figure 18. Phylogenetic reconstruction and comparison of YDA orthologs structure. A phylogenetic tree was constructed with MEGA 6 using the MAPK domain by the Neighbor-joining method and 1000 bootstrap value. Sequences were retrieved from the Phytozome database and TAIR.

MATGAT analysis of the three different domains present in the YDA protein showed a high percentage of similarity and identity between YDA and their orthologs. The two cucumber *YDA* genes display an overall 73% similarity with AtYDA, with the MAPK domain being the region with highest similarity (up to 90%) and identity (up to 80%) (Figure 19).
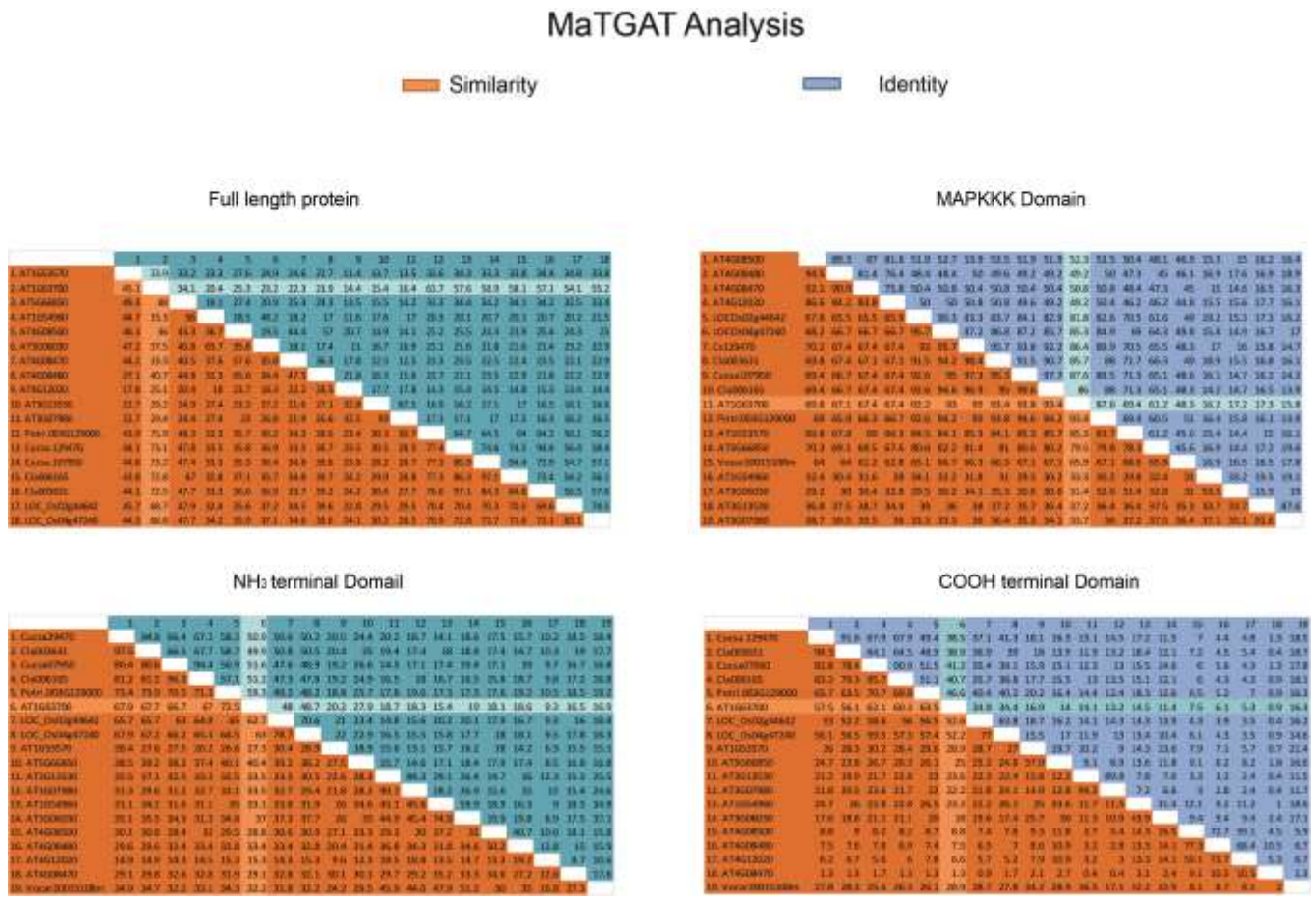
Figure 19. MaTGAT Analysis shows high percentage of identity and similarity of Arabidopsis YDA with YDA from Cucurbitacea.

The accession numbers of the cucumber genes are Cucsa.129470 and Cucsa.107950; to determine whether one of them, or both is an Arabidopsis YDA ortholog, but in different tissues, we decided to check their expression levels in cucumber leaves and vascular bundles. Here, actin 8 was used to normalize the data, and the results show that both genes are more expressed in vascular bundles, although the Cucsa107950 (107) transcript is more abundant in this tissue
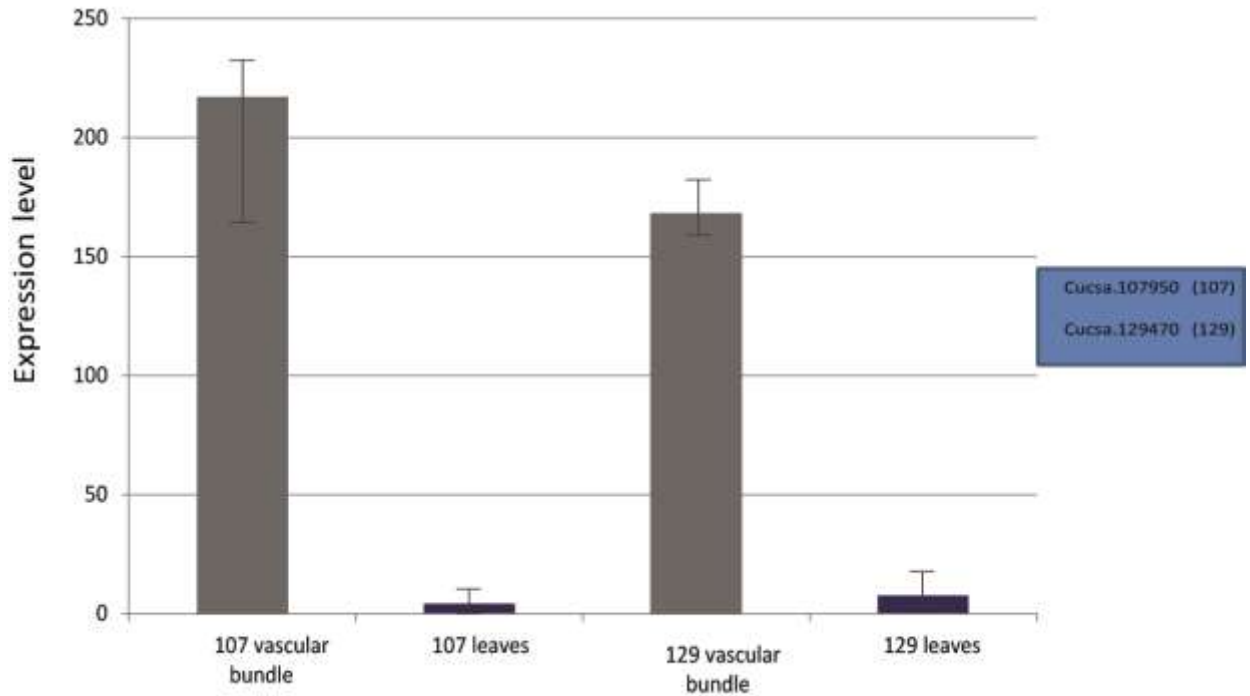
(Figure 20).

5.5.2 Cloning in the ZYMV vector

With the *in silico* results supporting the AtYDA expression in pumpkin, the protein was split to clone the three domains of YDA. First YDA N-terminal, C-terminal and MAPK domains were ligated to the pZero PCR blunt vector. After corroborating their presence in the plasmid, these fragments were ligated to ZYMV. Results of the cloning are shown in Figure 21. The N-terminal domain could not be cloned in the ZYMV vector since this vector does not accept more than 50 kDa.

Figure 20. *YDA* expression in vascular strips of cucumber. Both genes are highly expressed in vascular bundles.
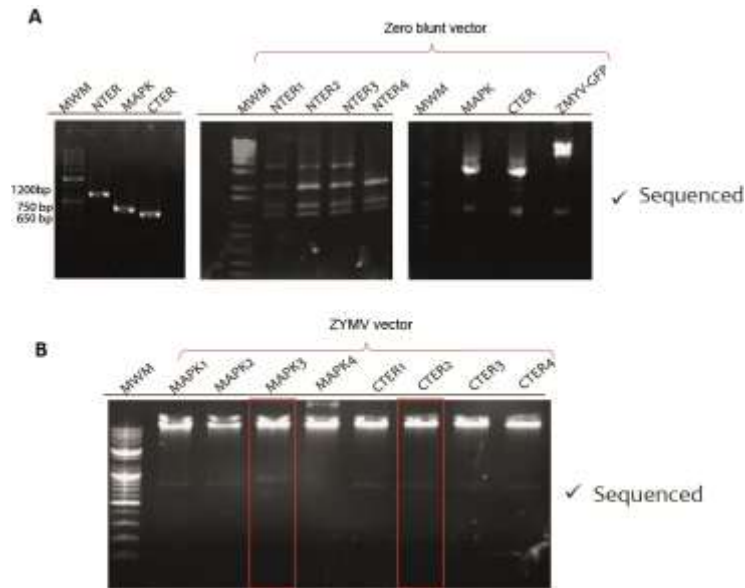
Figure 21. Amplification and cloning of *YDA* in ZYMV vector. A) Amplification of the three domains. Restriction pattern of the fragments in pZero Blunt TOPO. B) Fragment release after digestion with with *Sph*I and *Kpn*I in ZYMV.

The clones harboring the C-terminal and MAPK domains were confirmed by sequencing and then used to transform pumpkin seedlings by microprojectile bombardment. After 8 days post inoculation the leaves showed the characteristic yellow spots of ZYMV infection; small leaf discs were used to detect the protein (Fig 22).

GFP was also expressed in pumpkin as an immunoprecipitation control. After 8 dpi the protein was extracted and purified by Nickel affinity chromatography using 5 mM imidazole to avoid unspecified binding. The protein was correctly eluted and was detected in the elution fractions 7 and 8. These fractions were purified by immunoprecipitation with c-Myc antibody; the protein appeared in the c-Myc elution and the flow through (FT) as shown in Figure 23 (D and E).
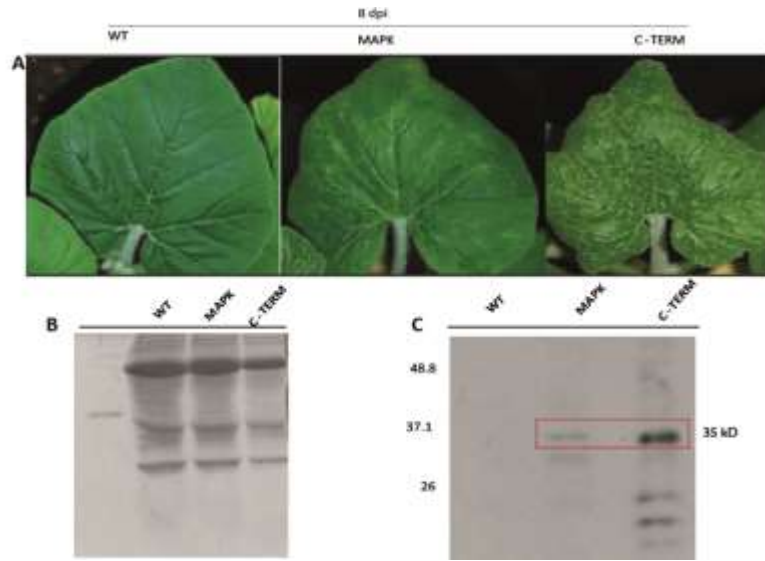
Figure 22. C-terminal and MAPK domain is detected by Western blot after 8 dpi. A) Leaves showing symptoms of ZYMV infection. B) Coommassie blue stained gel and C) Western blot by c-Myc shows 2 fragments of approximately 35 kDa.

The constructs harboring MAPK and C-terminal in ZYMV were transformed by biolistic. Both domains showed yellow spots characteristic of ZYMV infection and tissue sample was collected to detect the protein by Western blot. Then the harvested leaves were used for protein exraction and purify by affinity. The MAPK domain failed to adhere to the resin; most was detected in the flow through, but weak bands were observed in elution fractions 2 and 3 (Figure 23A). These fractions were loaded in a new gel to inspect these bands more closely. While there was cross reaction with the antibodies recognizing the epitope, the size of the bands was smaller than expected (Figure 23C). Despite this, fractions were immunoprecipitated, but the protein was not detected afterwards.
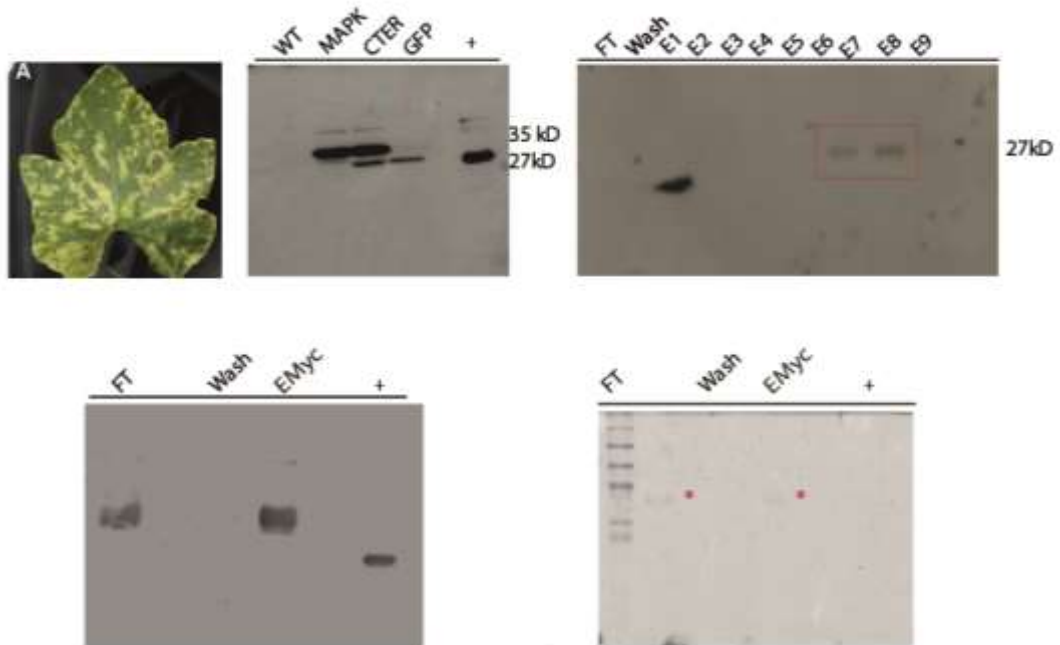
Figure 23. Purification of GFP expressed in pumpkin. A) Leaves with ZYMV symptoms. B) protein detection using c-Myc antibody. C) First purification of GFP by Nickel affinity. D) Western blot of GFP purified by immunoprecipitation. E) Coommassie blue staining showing a band corresponding to expected MW of GFP.

The C-terminal domain purification results were similar to those obtained with the MAPK domain. The protein did not bind to the column and was detected in the flow through and the washes. In this case no band was detected by Western blot in any elution fraction. Concentrated flow through was used for the second step of purification, although the protein could not be obtained from this fraction (Figure 24 D, E).
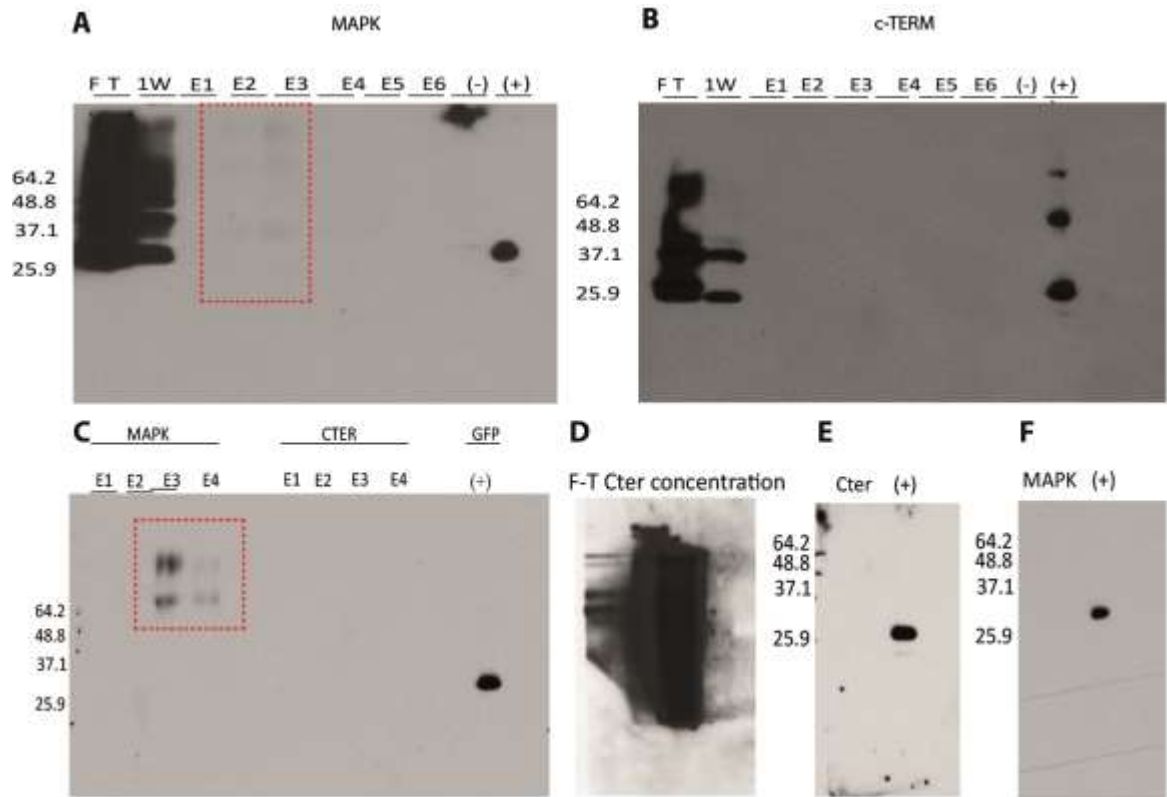
Figure 24. Western blot using c-Myc antibody. A) Affinity purification of MAPK domain B) Affinity purification of C-terminal domain. C) Second Western blot of both domains using elution fractions. D, E) Concentration of FT and Myc purification of C-terminal domain. F) Myc purification of MAPK domain. Positive (+): GFP.

5.6 Expression of Arabidopsis YDA in *E. coli*.

5.6.1 Cloning in pTrcHis 2 TOPO.

For co-immunoprecipitation assays, it is necessary to obtain the full-length protein. Since cloning proteins larger than 50 kD in ZYMV is difficult, expression in *E. coli* was used as an alternative for purification of YDA.

Arabidopsis *YDA* ORF without stop codon was amplified and directly ligated into pTrcHis 2 TOPO, which contains a Myc tag and 6 Histidines in the C-end. *E. coli* DH5α was transformed and resultant clones were selected by colony PCR. After amplification of a 200 bp *YDA* fragment, orientation was determined by digestion with *Eco*RV (Figure 25). After sequencing, the plasmids were introduced into *E. coli,* strain BL21, which is suitable for heterologous protein expression.
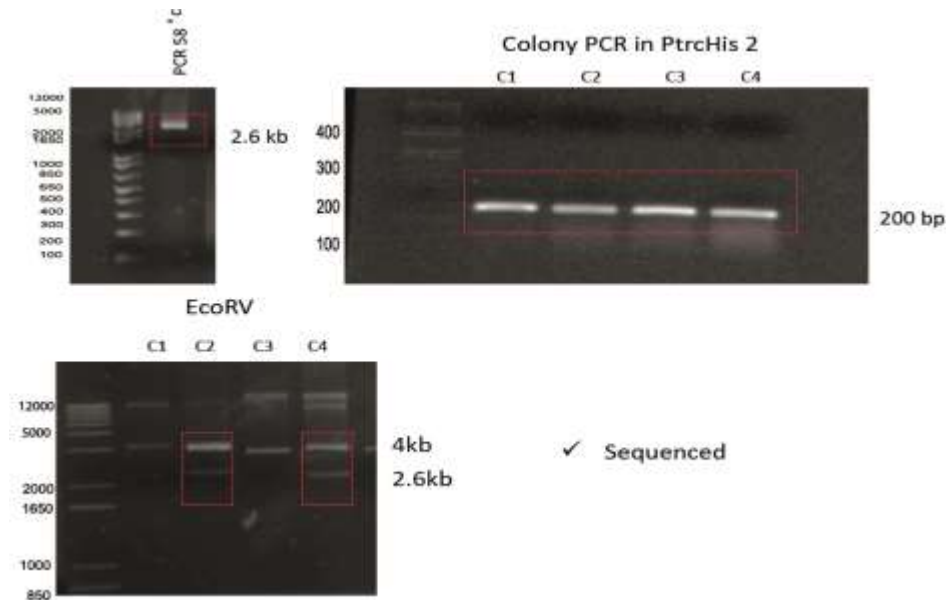
Figure 25. Cloning of *YDA* ORF in pTrcHis 2 TOPO. A) PCR of YDA ORF showing the product with the expected 2.6 kb size. B) Colony PCR showing 200 bp YDA fragment amplification products (sequences in supplemental information). C) Restriction pattern of these plasmids after EcoRV digestion.

BL21 strain was grown in TB medium and induced with IPTG. Samples were collected before induction and after 1, 2, and 3 h. Protein was detected in both soluble and insoluble fractions, although the size is close to the 46 kDa band of the protein marker (Figure 26A).

E. coli being a prokaryote organism may have problems expressing eukaryotic proteins because of different codon usage. The Arabidopsis YDA ORF was analyzed by the Rare Codon Calculator (http://nihserver.mbi.ucla.edu/RACC/) to determine whether it contains rarely used codons in *E. coli*. The results obtained showed 49 rare codons corresponding to arginine (Figure 26B). Thus, another

Figure 26. Expression of YDA in E. coli BL21 strain. A) Western blot showing an smaller protein than YDA that is recognize by c-Myc antibody. B) Rare Codon Calculator analysis of YDA ORF showing the location of arg rare codons, decreasing its correct translation.

strain known for rare codon usage was used. Indeed, *E. coli* Rosetta 2 harbors a plasmid that allows expression of eukaryotic proteins. Rosetta 2 clone was grown in TB supplemented with chloramphenicol and carbenicillin to account for vector pTrcHis2 resistance. In contrast to BL21, full length YDA protein was expressed in Rosetta 2. After 1 hour of induction the protein was detected in the soluble but not the insoluble fraction. After 2 hours the amount of protein increased but the amount in the insoluble fraction increases as well (Figure 27 A). After 3 hours induction there were no traces of YDA protein, therefore the purification assays were performed
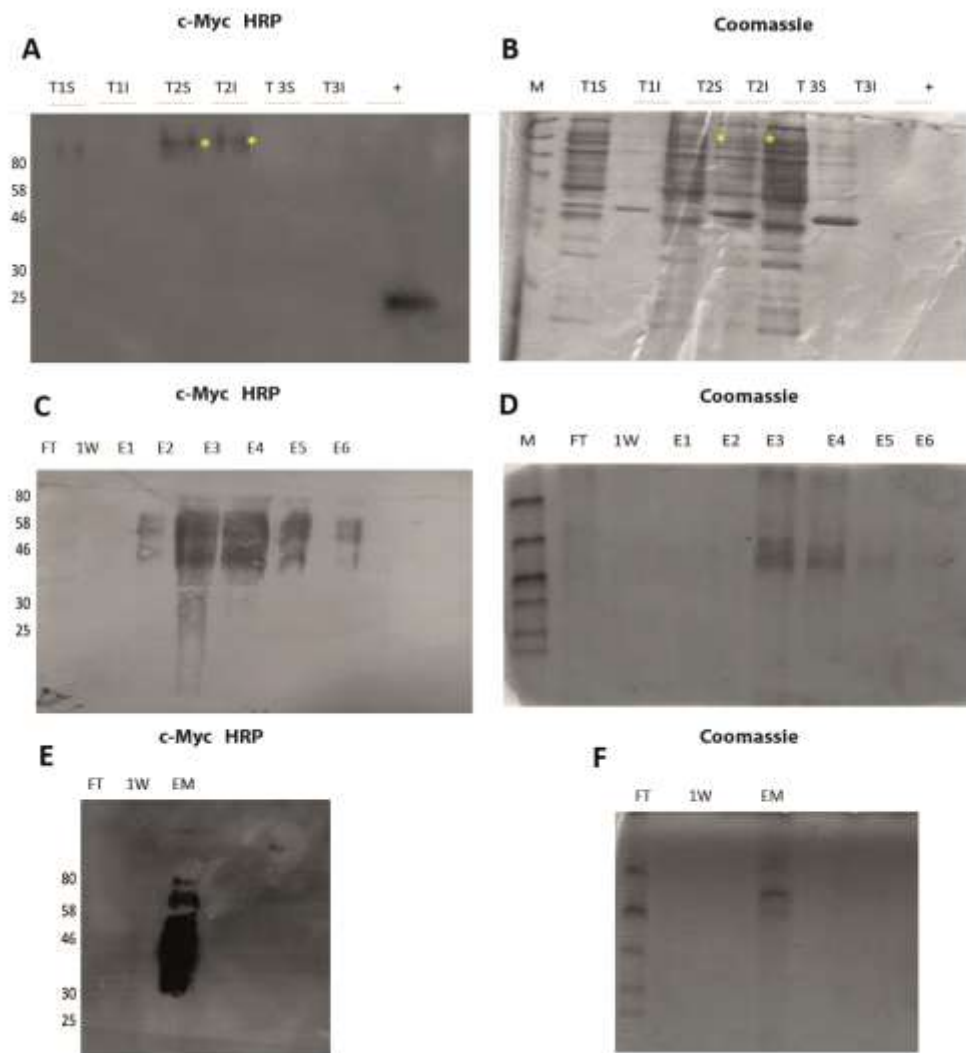
Figure 27. Expression of YDA in Rosetta 2 strain. A, B) Western blot and Coomassie blue staining gel showing the induction of YDA from 1- 3 hours. C, D) Western blot and Coommassie blue staining gel of affinity purified proteins; the fragment size belongs to a smaller protein than YDA. E, F) c-Myc inmunoprecipitation of E3 and E4 from affinity purified proteins.

2 hours after induction when the protein was still detectable. In order to improve purification by affinity, cobalt was used instead nickel on the column.

The sample passed through the cobalt column. The retention of the protein was better than with nickel but the bands recognized by the antibody do not correspond to YDA size, instead bands smaller than 60 kD were observed (Figure 27 C, D).

YDA bands that cross-react with the anti c-Myc antibody appear smaller than the expected size, but nonetheless the fractions containing these fragments were purified by immunoprecipitation with the aforementioned antibody. The Myc elution fraction was positive by Western blot for the tag, but the size does not correspond to full length YDA (Figure 27 E, F).

Precipitation with ammonium sulfate was performed previous to the first purification for YDA enrichment in the sample. A concentration gradient was done starting with 20% until saturation was reached at 85%. The 96 kDa protein was present in the 40% pellet, as well as the smaller ~60kDa protein. After 50% only the small protein was enriched (Figure 28 A, B).The pellet
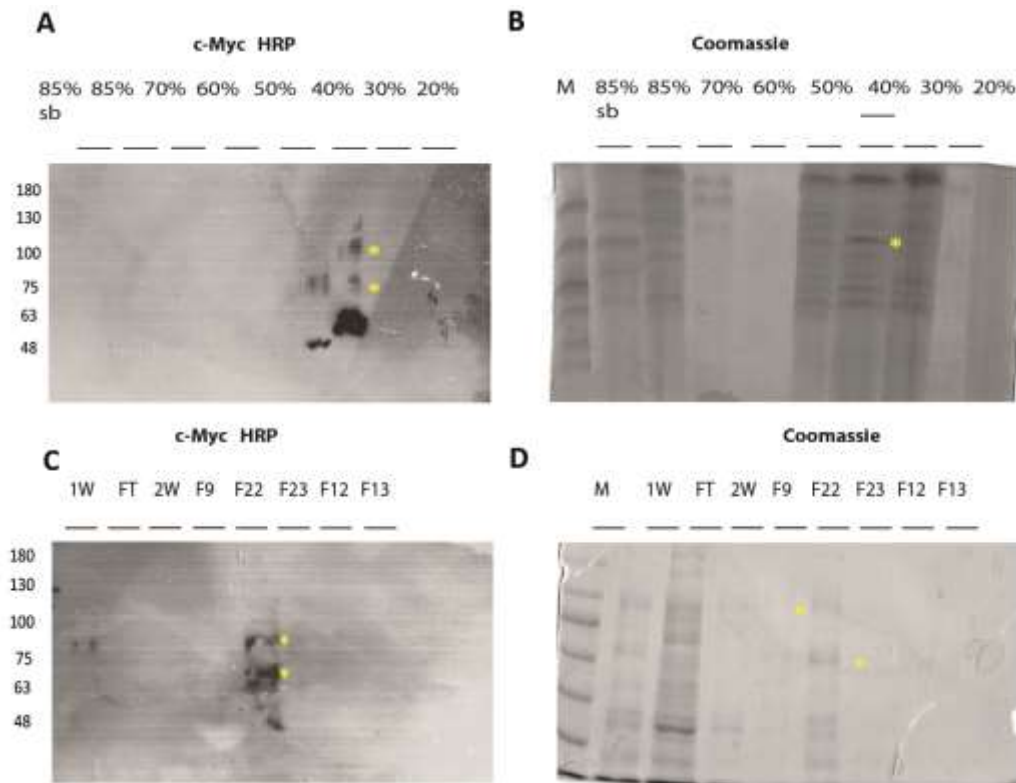
Figure 28. Ammonium sulfate precipitation and Affinity purification of YDA. A), B) Western blot and Coommassie gel staining showing YDA protein in the 40% fraction. C, D) Western blot and Coommassie gel showing the protein profile in the fraction 22.

containing YDA was dialyzed against the extraction buffer for salt removal; then, the first purification step was done using FPLC Äkta pure. Fractions with a 280 nm absorbance peak were examined in a polyacrylamide gel (Figure 28 C, D). Fraction 22 cross-reacted with c-Myc antibody but the smaller protein is more abundant and it has higher affinity towards the resin.

5.7 Agroinfiltation of *N. benthamiana* plants with ORF YDA-GFP

YDA transient transformation was also performed using a construct of pEarley gate 103 vector containing YDA. Agroinfiltration was performed essentially as described by Leuzinger *et al.* (2013) using *N. benthamiana* plants 6 weeks after germination. Leaves were vacuum infiltrated and after 5 days leaves were observed under a hand-held long-wave UV lamp to monitor GFP expression (Figure 29 B-D). The protein was detected by Western blot using as controls samples from *YDA*-overexpressing Arabidopsis plants (Figure 29 E).
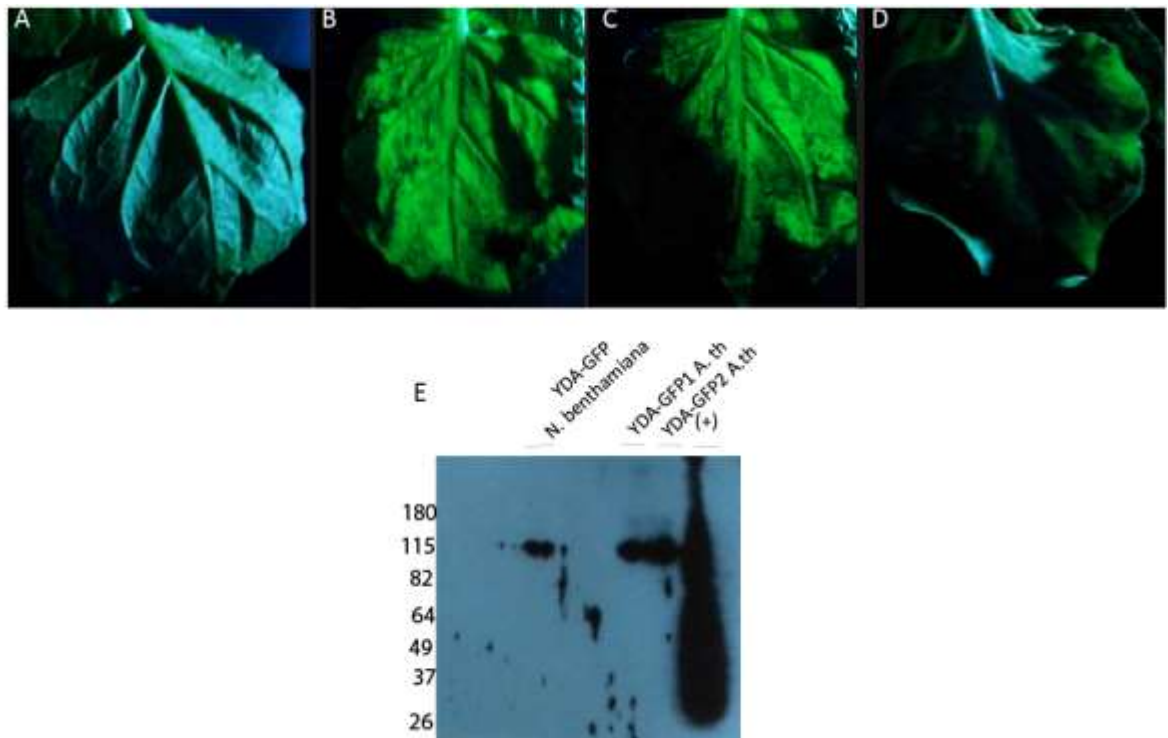
Figure 29. Agroinfiltration of *Nicotiana benthamiana* with *A. tumefaciens* harboring *YDA-GFP.* A) Mock-infiltrated leaf under UV lamp illumination. B-D) Agroinfiltrated leaves under UV lamp. E) Western blot detection of YDA in agroinfiltrated plants.

5.8 Phylogenetic Analysis of proteins involved in vascular tissue differentiation and/or function

Previous work in Arabidopsis showed that *YDA* upstream sequences, as well as other genes, the RNAs specified by which were found in the pumpkin phloem transcriptome of sap, harbor specific motifs ihat could be involved in their expression in vascular tissue (Ruiz-Medrano *et al.,* 2011). Promoters of the homologous genes in vascular and non-vascular plants, as well as chlorophytes, were analyzed, showing that these motifs could be an important factor in coordinating their expression in this tissue (Martínez –Navarro *et al.,* 2013).

Phylogenetic reconstructions of some proteins that may be involved in phloem development and function were performed, namely APL, OPS, YDA, FT and CmPP16. For APL phylogenetic analysis was done using maximum likelihood. This protein is present from chlorophytes to higher plants.
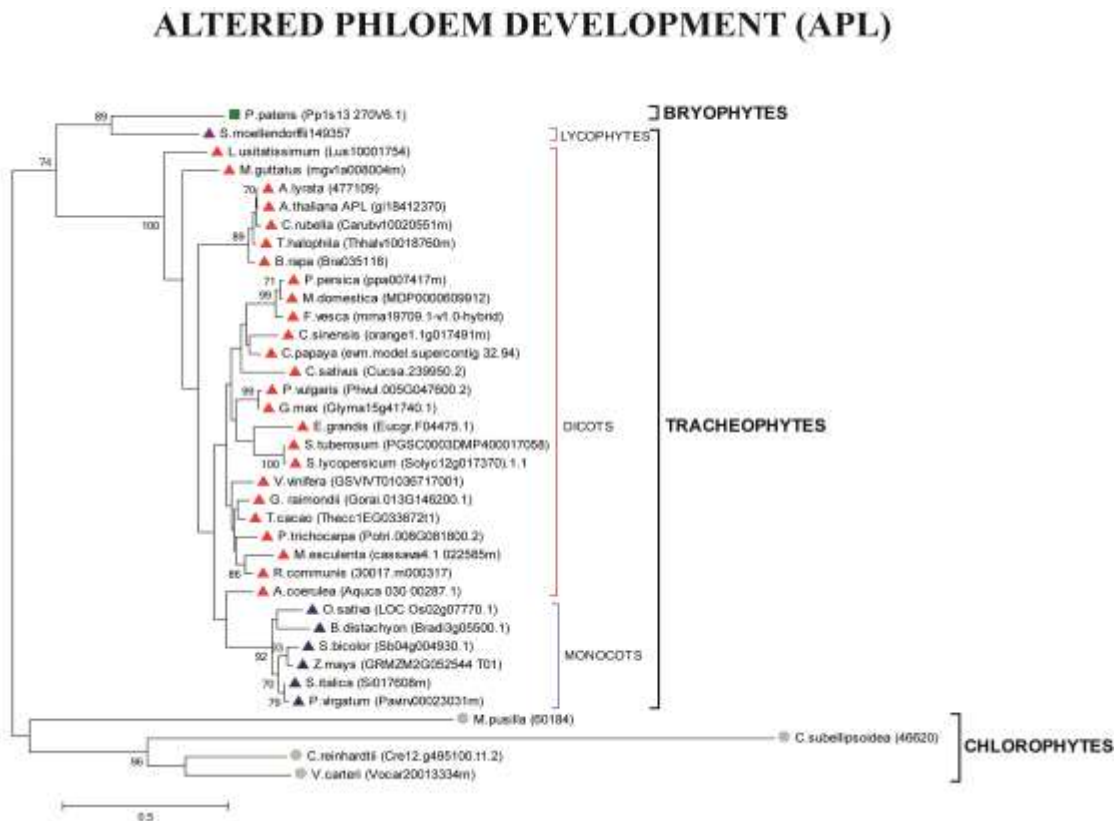
Figure 30. Phylogenetic tree of APL by Maximum likelihood. *C. subellipsoidea* was used as outgroup. Martínez-Navarro *et al.* (2013).

According to the phylogeny, APL phylogeny reflects that of the viridiplantae, in which three clades are evident: chlorophytes, tracheophytes and bryophytes (Figure 30).

OPS protein homologs were not found in *Physcomytrella* and chlorophytes. The phylogeny for this protein family was done by the Neighbor-Joining method. Since its presence is restricted to vascular plants, *Sellaginella moellendorffii* was used as an outgroup in order to construct this phylogeny. Again, the phylogeny based on OPS reflects the known evolutionary relationships between plant clades. Indeed, monocots and dicots form two separate clades (Figure 31). Next, a phylogeny for YDA was done also using the Neighbor-joining method.
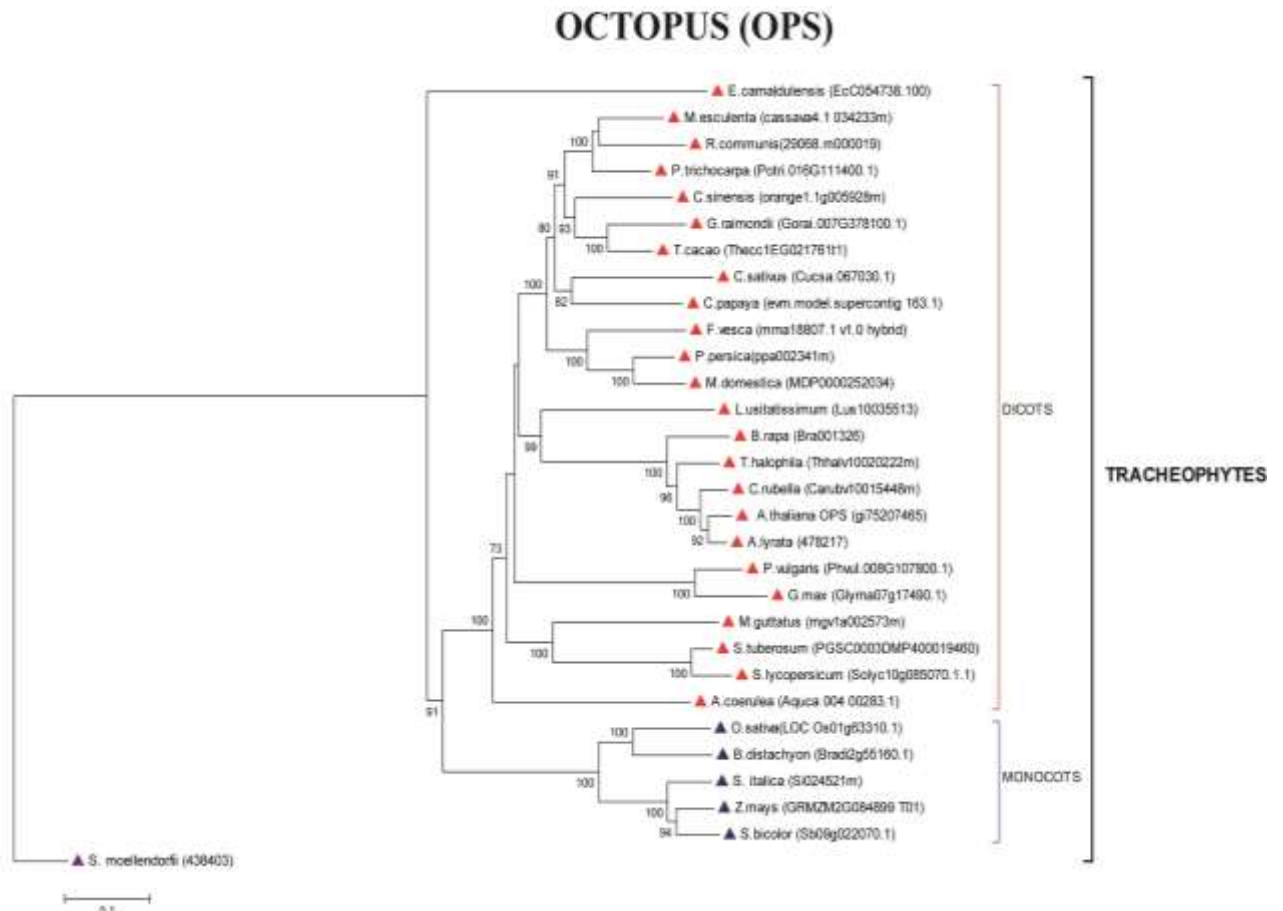
Figure 31. Phylogenetic tree of OPS by Neighbor-Joining with a 1000 bootstrap value. *Selaginella moellendorffii* was selected as outgroup.

Only one representative homolog per taxon was used to construct the YDA phylogeny. The monocots grouped according to their known evolutionary history; however *Carica papaya* YDA does not group within the eudicot clade. Instead, it appears more related to chlorophyte sequences. The majority of YDA homologs have a similar size, which is also according to their phylogenetic proximity to AtYDA, although the length of the *Carica papaya* sequence is smaller than the other dicot YDA (Figure 32).
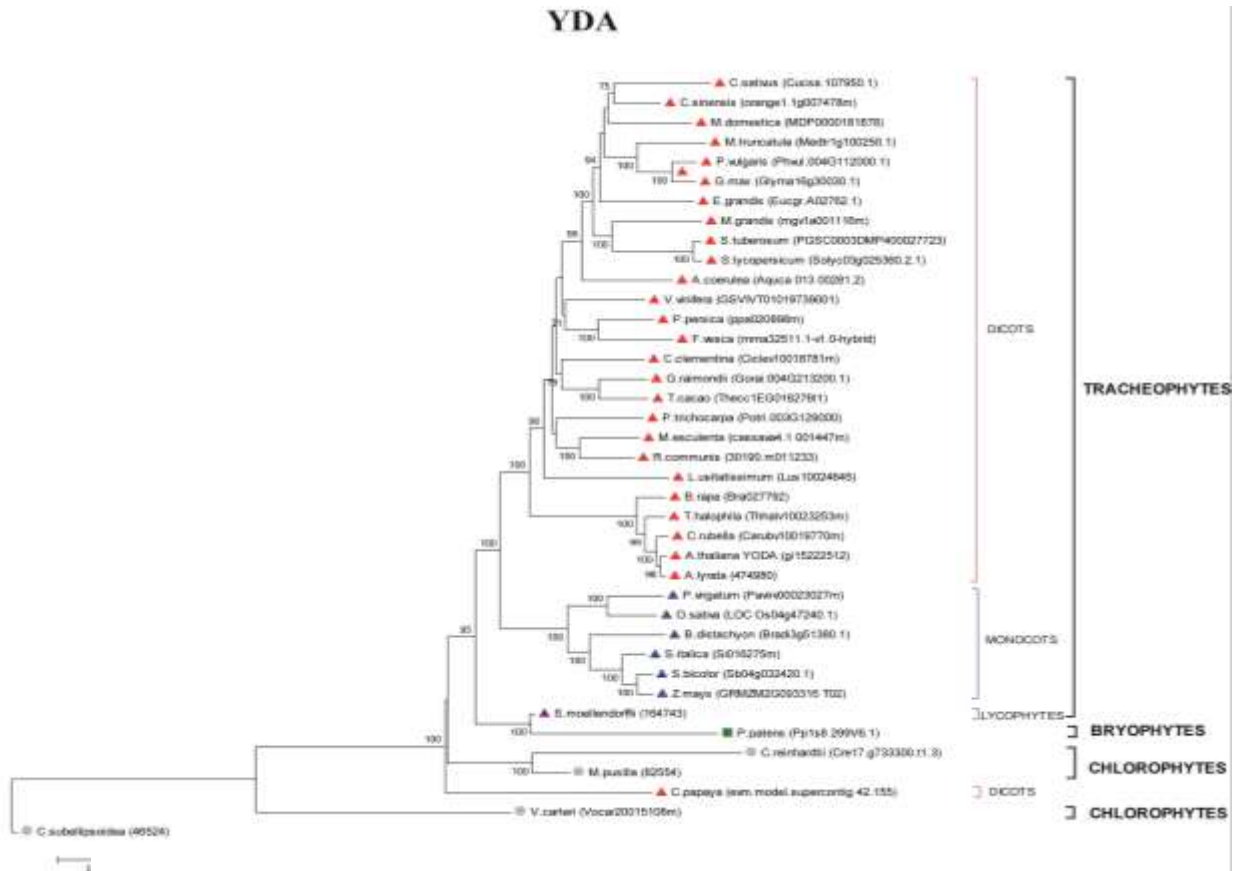
Figure 32 Phylogenetic reconstruction of YDA orthologs by Neighbor-Joining. *C. subellipsoidea* was used as outgroup. The *Carica papaya* YDA does not group in the dicot clade.

FT (FLOWERING LOCUS-T), which is a protein transported via the phloem from mature leaves to the apex, where it induces flowering. This protein groups according to land plant have arisen showing a species tree. The phylogeny was done by Maximum likelihood, being the best method for this protein. As with APL and YDA, FT is found also in *C. subellipsoidea*, but intriguingly no homologs were found in the other chlorophytes analyzed, although this could be readily explained if an incomplete annotation in other algal lineages is invoked (Figure 33).
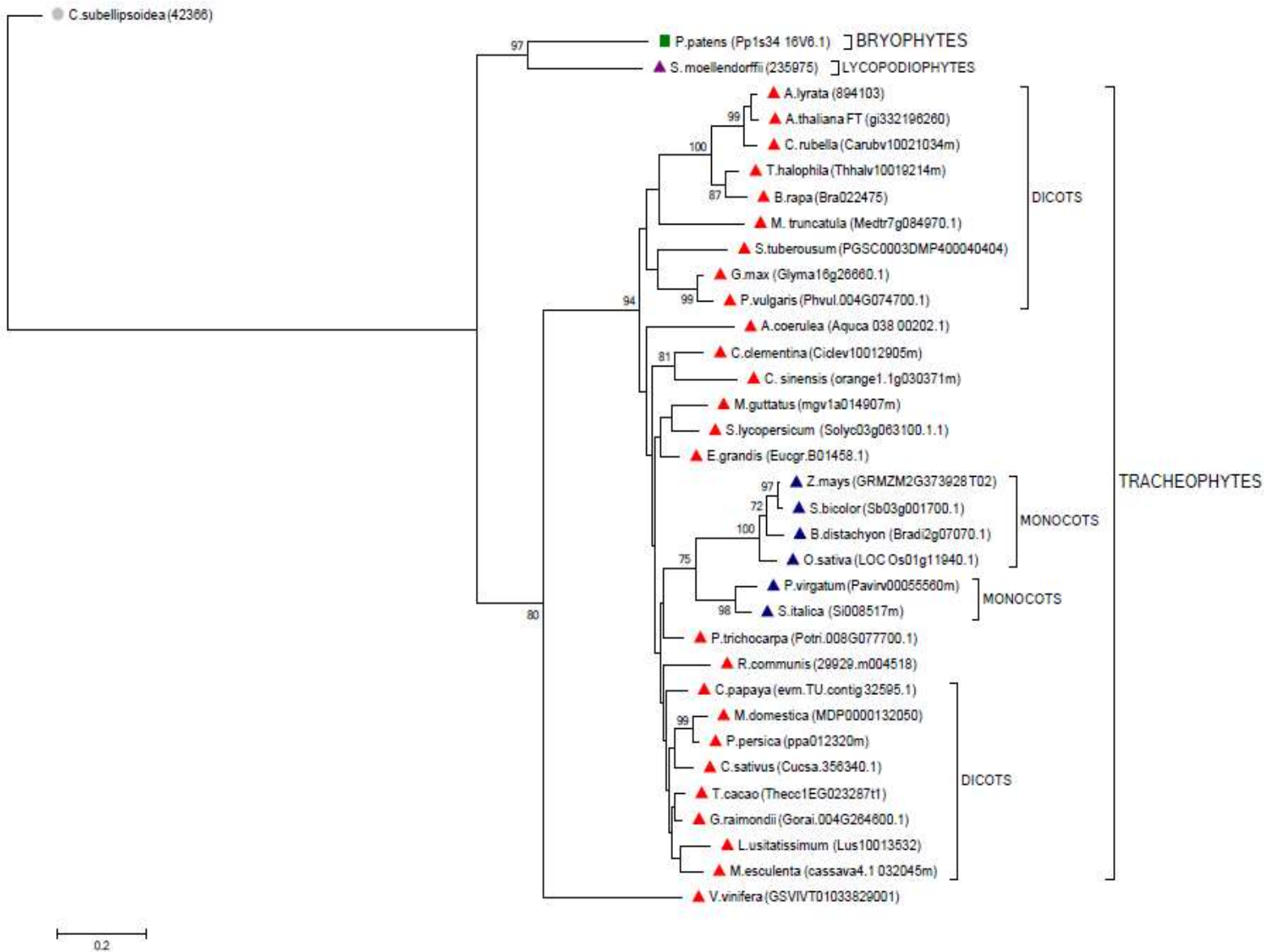
Figure 33 Phylogenetic reconstruction of FT by Maximum likelihood. C. subellipsoidea was used as outgroup; this was the only ortholog found in chlorophytes.

CmPP16 is an RNA-binding protein that moves long distance within the pumpkin phloem translocation stream, although its role in phloem function is not clear (Xoconostle-Cázares *et al.,* 1999). It has been found in all plant lineages, however a larger version that corresponds to synaptotagmin (a protein involved in vesicle trafficking, initially described in synaptic vesicle transport) is the only form found in chlorophytes. The selected method for this protein was Maximum likelihood. In contrast with the other proteins analyzed its phylogeny does not follow a species tree. *C. subellipsoidea* was used as outgroup and is distant from the other algae. *O. lucimarinus* groups with *Physcomytrella patens*, which is a bryophyte. *L usitatissimum* CmPP16 forms an isolated clade separated from the other dicots. *M. guttatus* CmPP16 is further separated from the dicot lineage. Interestingly, the *S. moellendorffii* protein is more closely related to CmPP16 (Figure 34).
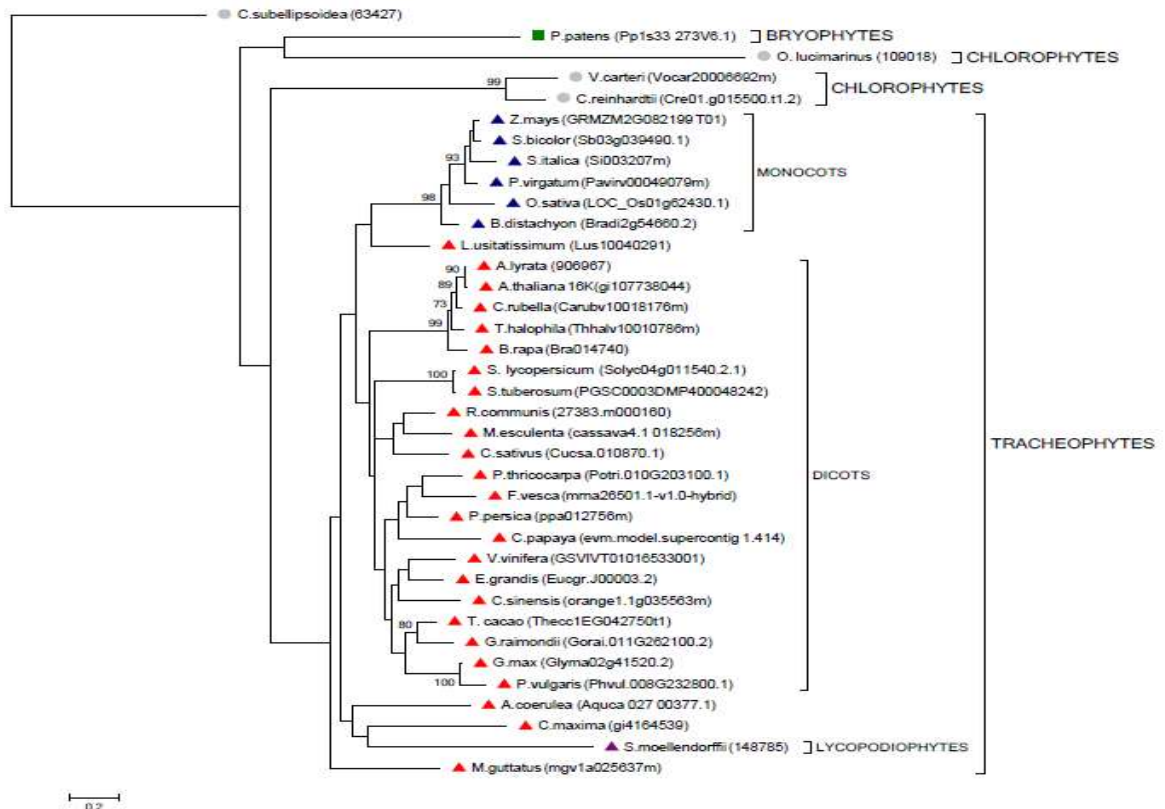
Figure 34. Phylogenetic reconstruction of CmPP16 by Maximum likelihood. *C. subellipsoidea* was used as outgroup; this was the only homolog found in chlorophytes.

DISCUSSION

6 DISCUSSION

The MAPKKK YDA (YODA) is a component of a signaling pathway that has been thoroughly studied, and has been reported to have important roles during different developmental processes. These roles include promotion of suspensor cell elongation, controlling stomatal density in leaves, directing morphology of floral organs, altering cell division orientation plane, and affecting primary and lateral root morphogenesis. (Lukowitz *et al.*, 2004; Bergmann *et al.*, 2004; Meng *et al.*, 2012; Bemis *et al.*, 2013; Smékalová *et al.,* 2014) (Figure 34). All the above are accomplished by a process called asymmetric cell division, in which YDA plays an essential role. On the other hand, the vascular tissue is also formed through this process, procambium divides periclinally until it forms pericycle and vascular primordium; it then differentiates into vascular procambium tissue to form xylem and phloem (Elo 2009). Indeed, phloem formation involves asymmetric divisions that occur to form sieve elements and companion cells. Whereas many genes associated with xylem formation are known (Motose *et al.,* 2001, Motose *et al.,* 2004, Kubo *et al.* 2005, Fukuda *et., al* 2004), just a few are reported for phloem formation (Bonke *et al.,* 2003, Truernit *et al.,* 2012, Anne *et al.,* 2015) The results obtained in this work suggest that YDA could have a role in the formation of vascular tissue. Since the ontogeny and patterning of the vasculature differs in the various tissues and developmental stages and due that the bulk of studies on YDA function are restricted to early stages of development and tissues thus limiting the knowledge of YDA function. We decided to focus on studying the function of YDA in vascular tissue during later developmental stages.
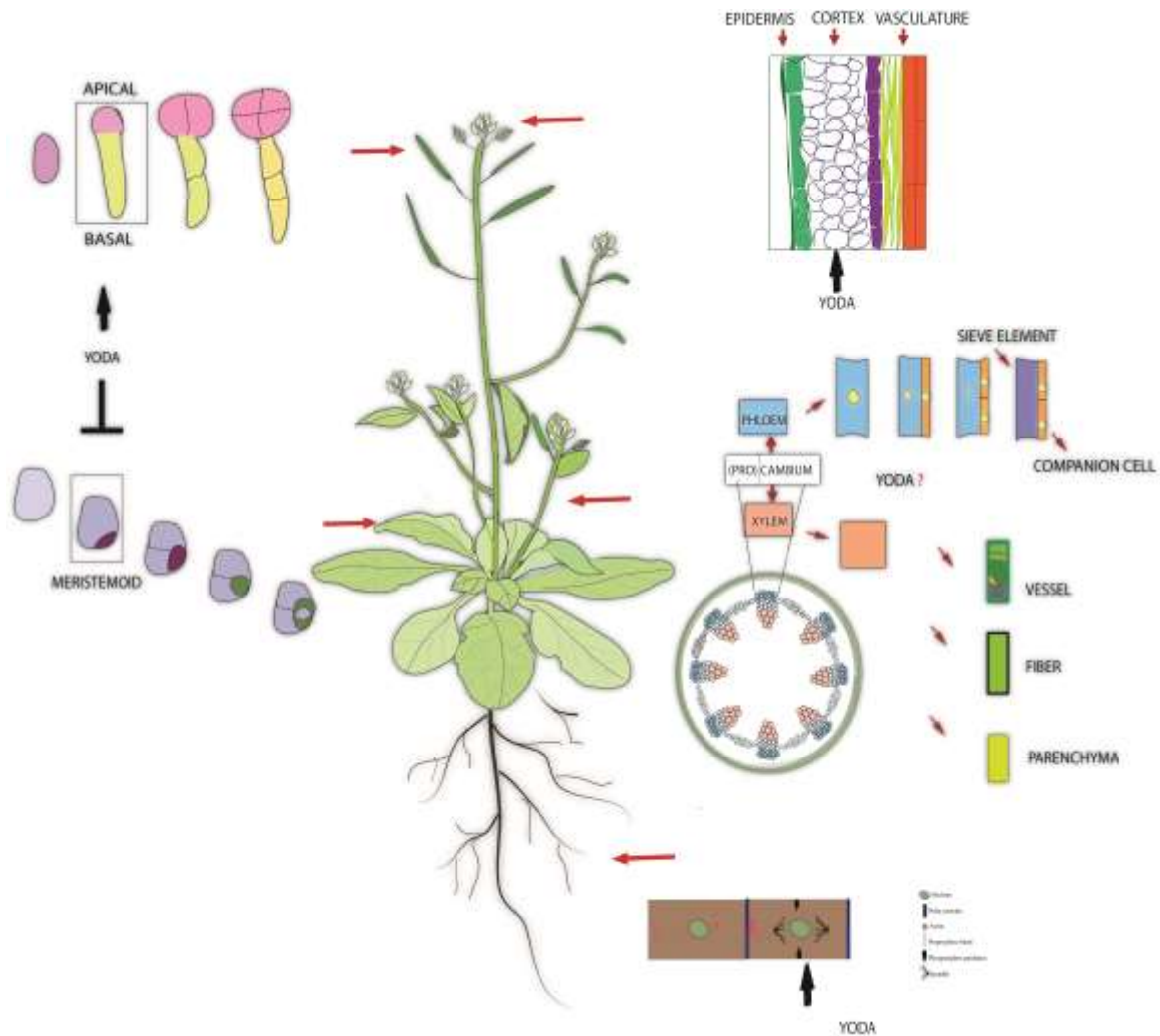
Figure 35. YDA participates regulating asymmetric cell division in zygote, stomata, inflorescence and roots.

6.1 YDA is expressed in growing tissues and in vasculature of different organs.

YDA mRNA accumulates in all tissues at low levels, as revealed by Northern blot analysis (Bergmann *et al.,* 2004). More importantly, its gene promoter directs expression of GFP in vascular tissue in veins of mature rosette leaves (Ruiz-Medrano *et al.,* 2011). Our results show that the expression pattern in leaves varies between rosette and cauline leaves; in rosette leaves *YDA* is expressed in the central vein as well as in the secondary veins while its expression in

cauline leaves is restricted to secondary veins and epidermis, also YDA is highly expressed in axillary buds.

Consistent with the literature *YDA* is expressed in floral organs and siliques in agreement with the reported function of YDA in inflorescence and silique elongation process (Meng *et al.,* 2012). We also noticed striking coincidences between *YDA* and *ERECTA* expression patterns, a known receptor upstream of YDA in the stomata formation pathway. These proteins are both present in phloem and xylem cells as well as in epidermis of inflorescence stems. Moreover, we noticed that *ERECTA* is also expressed in carpel, in a report originally focused on its expression in pedicel and stem (Uchida *et al.,* 2012). Our results confirm *YDA* expression in carpel in mature flowers, similar to the *ERECTA* expression pattern. These overlapping expression patterns suggest that ERECTA could be acting upstream of YDA in vascular tissue.

Confocal microscopy allowed us to observe that the protein also accumulates in phloem cells. GFP is found in sieve elements; however, green fluorescence signal is also intense in xylem, which was higher than WT controls, suggesting a role for YDA in this tissue. However, these data must be interpreted carefully, since the xylem is very autofluorescent. It is necessary to analyze more tissues to confirm if the expression pattern is consistent with protein accumulation. Even if these do not overlap completely, protein and/or mRNA movement of YDA cannot be ruled out.

6.2   *YDA* mutants display a severe phenotype in vasculature of inflorescence stem.

Evaluation of YDA mutants showed that this protein plays an important role in stem vasculature patterning non-lethal YDA mutants were obtained by mutagenesis with EMS (Lukowitz *et al.,* 2004). *yda-1* is a nonsense mutant generating a truncated protein in the catalytic domain. This feature results in 10% of survival due to the correct elongation of the zygote. Despite that the

emb71 mutation site has not been characterized, the mutant is reported as *yda-1* like (Meinke *et al.,* 2009).

 The study of these mutants has been hampered because of the low germination and survival rate, which was confirmed during our analysis, at least under the conditions tested in the present work. As *YDA* null mutations are lethal, heterozygous plants were used to analyze the structure of inflorescence stem. These plants displayed an abnormal phenotype on this tissue. The stem shape is distorted compared to WT, and also smaller. These mutants display less vascular bundles, the arrangement of which also appears distorted.

Vascular patterning is controlled by HD ZIP III and KANADI genes (Bowman *et al*; 2002, Emery *et al*., 2003). Mutations in these genes show defects in leaf vascular tissue, which is not unexpected since they also have a role in stem vasculature arrangement (Emery *et al*., 2003). Mutants in *REV* and the triple mutant *kan1, kan 2, kan 3* showed altered stem shape; also, an amphivasal arrangement with xylem cells surrounding phloem. The organization and symmetry of the vascular bundles in these mutants resemble the organization observed in *YDA* mutants.

The vascular bundle of these mutants appear flattened with decreased number of phloem and cambium cells, similar to other mutants, such as mutants in the *PXY/TDR* receptor. These display a flattened vascular bundle, although the difference is that *PXY* expression is confined to procambial cells (Fisher and Turner 2007). The flattened phenotype is due to phloem cells intercalated with xylem, which can be observed by aniline blue staining. The notion that the function of these genes, along with *YDA*, converges on vascular bundle arrangement is supported by comparison of the respective phenotypes (see Figure 35).
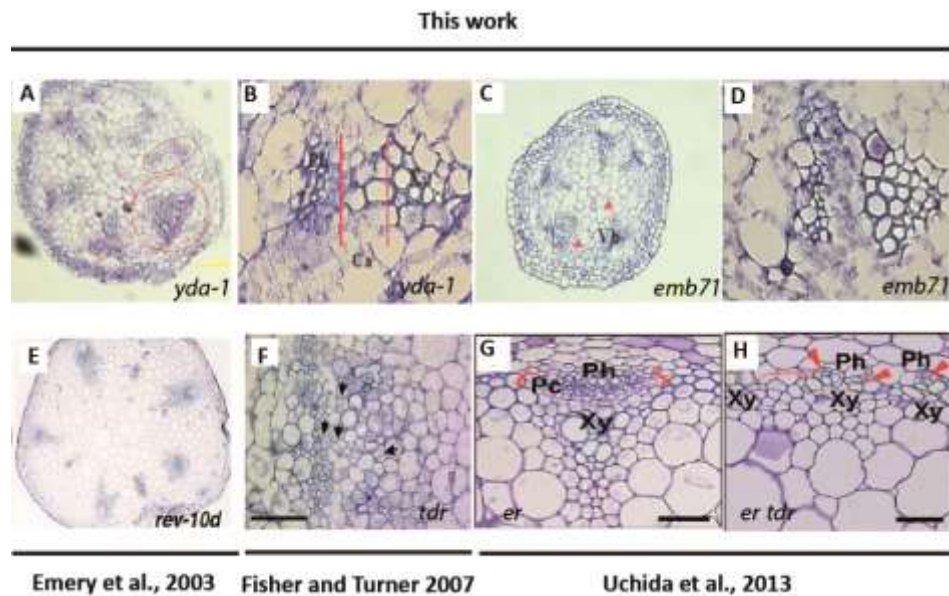
Figure 36. Comparative image of inflorescence stem *from yda-1 and emb71 (this work) and other reported mutants.*

6.3 YDA is transcriptionally regulated by SA and Citokynin.

The response of *YDA* to hormones had been tested in previous works to know whether addition of exogenous growth regulators could reestablish the WT phenotype in *yda* mutants, but none of the hormones used had effect on these plants (Lukowitz *et al*., 2004). Phenotype rescue by addition of growth regulators can be helpful if they are downstream of YDA, but are not useful in elucidating their role in this pathway if they act upstream of YDA.

Kim *et al.* (2012) reported that YDA is regulated by the GSK3 protein BIN2, which is repressed by brassinosteroids. This pathway appears to be parallel to the stomatal development cascade. Similarly, it was found that YDA participates in the elongation of primary and lateral roots via auxin up-regulation (Smékalová *et al.,* 2014). Although this modulation is at the protein level there is also evidence of transcriptional regulation of this gene in stomata to confer drought tolerance (Meng and Yao, 2015).

In this work we performed qRT-PCR assays to determine whether *YDA* mRNA levels are affected by exogenous addition of different growth regulators. According to the results presented

here, the transcript levels, likely *YDA* transcription, are repressed in both seedlings and leaves in adult plants by most of the hormones tested. Plants accumulate salicylic acid (SA) in response to biotic and abiotic stress, which triggers Systemic Acquired Resistance, and programmed cell death induced by ROS in the case of hypersensitive response (HR) (Gaffney,1993; Overmyer *et al.,* 2003; Blanco *et al.,* 2005). SA accumulates in local and systemic tissues, where it induces different defense responses; in the case in which the pathogen harbors an avirulence gene, and the host the corresponding resistance gene, an HR that will induce cell death around the infection zone ensues (giving rise to plant immunity). Then, a phloem-mobile signal, the nature of which is still a matter of debate, is transmitted to distant tissues promoting the SAR and the secondary oxidative burst (Blanco *et al*., 2005).

NPR1 is a positive regulator of SA biosynthesis. MAPK3 /6, which are downstream of YDA in the stomata, embryo and root developmental pathways, regulate NPR1 activity during stress response (Blanco *et al*., 2005, Wang *et al.,* 2007, Meng *et al.,* 2015). Additionally, these MAPKs regulate PR5 and *PR1*/*NPR1* expression during leaf senescence, and also *WRKY* genes that are an important part of the defense response (Chai *et al.,* 2014; Robatzek and Somssich, 2002). The factors upstream of MAPK3/6 are still unknown, although recent studies with *YDA mutants* showed that this could also be involved in plant immunity and suggesting that the N-terminal domain of YDA could be the responsible for this response (Sopeña-Torres, PhD thesis 2015). Therefore it could be hypothesized that there is a feedback loop between SA synthesis and the YDA pathway. This protein might regulate negatively biosynthesis of SA; and in turn high levels of SA could downregulate *YDA* expression via transcriptional regulation in order to stop uncontrolled HR spread induced by external stimuli. It is also known that high concentrations of SA reduce transpiration and control pod number, increasing yield in *Phaseolus vulgaris* (reviewed in Raskin 1992)*.* Since there are several routes depending on the kind of stimulus (biotic or abiotic), more studies are necessary to know in the pathway in which SA controls YDA.

Abscisic acid (ABA) is a phytohormone involved in abiotic stress response, where it promotes stomatal closure, inhibits growth, and is involved in seed dormancy (Okamoto *et al.,* 2013). In this work it was observed that low concentrations of ABA repressed *YDA* transcript accumulation in stem and apex. ABA can be synthesized by guard cells; hormone levels regulate ABA

synthesis, so this response allows stomatal closure in response to low humidity (Bauer *et al.,* 2013). Its known that SA is antagonizes ABA-mediated growth inhibition (Raskin, 1992), YDA could be involved in some feedback loop between SA and ABA signaling given that SA induces their expression and ABA controls its transcription, although since ABA controls stomatal closure, YDA could act in a parallel pathway to regulate stomata closure during abiotic stress, and controlling stomata density during biotic stress.

Also, it is important to highlight negative regulation of *YDA* in stem and apex. ABA is a root-derived signal that is translocated to mature leaves via the transpiration route to reach its site of action (Seo and Koshiba *et al.,* 2011); it could inhibit growth by secondary signals that move to distant tissues via the phloem, especially in apex, or target specific processes, such as vasculature development. Indeed, GA and ABA are antagonist in roots in response to salt stress (Duan *et al.*, 2013).

It is important to take into account the crosstalk of stress phytohormones with other hormones such as auxin, brassinosteroids, and cytokinin. Elements from the brassinosteroid pathway have been reported controlling stomata formation (Kim *et al.,* 2012) as well as protophloem differentiation (Anne *et al.,* 2015). Regarding stomata formation, it has been observed that BIN2 controls YDA function in absence of brassinosteroids at the posttranslational level, although in the present experiments we found that YDA could be also regulated by this phytohormones at the transcriptional level in inflorescence stem, having the opposite effect in leaves. The defects in vasculature displayed by the mutants correlated with vascular cell formation and patterning, which suggests that OPS could be upstream of the YDA MAPK pathway and could involve also BIN2 regulation.

In the case of cytokinin treatments, we observed that *YDA* transcript accumulation was repressed at the seedling stage. Cytokinins are key regulators of cell division, although they also have a role in apical dominance, leaf senescence, production of anthocyanins and sink/source relation (Hutchinson and Kieber, 2002). The hypocotyl and root elongation are directly related to cytokinin regulation. Benzyladenine (BA), an artificial cytokinin, inhibits the elongation of the hypocotyl in dark-grown seedlings, and also causes formation of curly apical hooks (Cary *et al.,* 1995). Lateral root formation in *YDA* loss-of-function mutants is affected; these show short roots

and excessive lateral root formation. In contrast gain-of-function plants present roots with curly ends and decreased number of lateral roots (Smékalová *et al.,* 2014); however, these authors analyzed only whether auxin accumulation could be affected by YDA. It is possible that there is also cytokinin regulation involved in the aforementioned *YDA* phenotypes; another important point is whether the regulation is at the transcriptional and/or posttranscriptional level. Auxin also affects *YDA* transcript levels in leaves of adult plants, although these assays as well as others in adult plants presented here were affected by the normalization with the endogenous gene polyubiquitin 10 (UBQ10). In contrast to adult plants during seedling stages UBQ10 expression was stable under all treatments. This gene has been reported as good normalizer for quantification of expression levels (Weigel and Glazebrook, 2002); however, in some conditions, such as abiotic or biotic stress, its levels could change. UBQ10 is induced during senescence, triggered by auxin, ethylene and fungal infection (Blanco *et al.,* 2005). The expression of this gene was similar regardless of the developmental sample with the exception of seeds; it is thus necessary to find genes that can be used as endogenous controls in different experimental conditions for expression analysis.
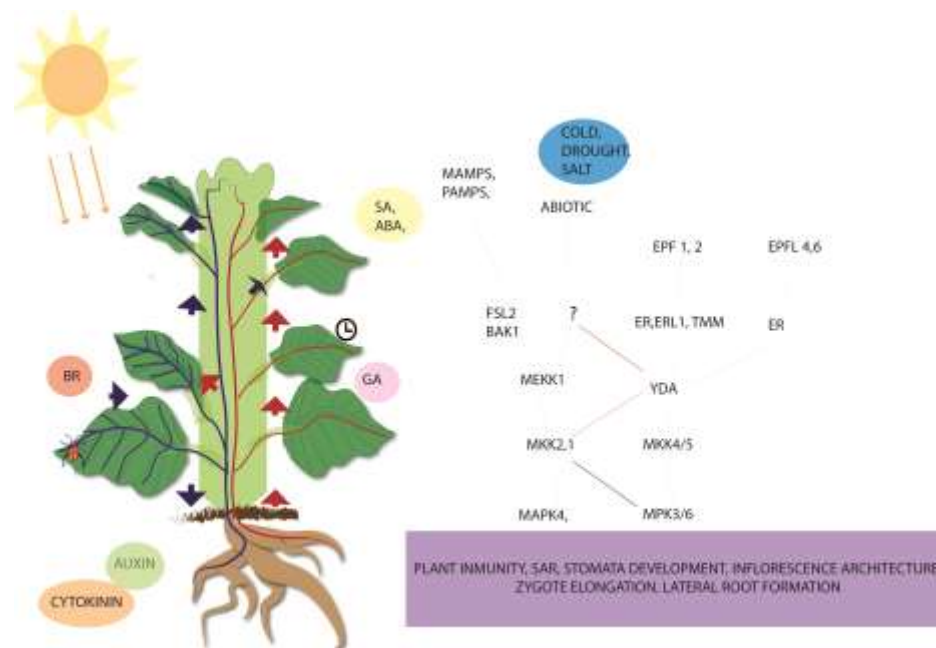
Figure 37. **A model depicting the potential roles of** *YDA* **in development, plant immunity and SAR and its crosstalk with other pathways.**

6.4   YDA protein instability complicated its purification

In order to purify YDA to carry out coimmunoprecipitation assays, which in turn will help find proteins that interact with YDA in vivo. Different expression protein assays were performed using different systems. The expression of the full-length protein in pumpkin was affected by the size of the protein because it is not possible to clone such a large protein into the ZYMV vector. The MAPK domain and C-terminal were successfully expressed in this system; however, the purification of these proteins faced problems because the fragments could not bind to the resin, avoiding the correct separation from the non-specific proteins. It is possible that the structure of these fragments could be affecting the exposure of the His tag.

For expression of the full-length protein we used *E. coli*. The Rosetta 2 strain expressed the protein correctly but only after 2 hours after induction. However, stability of the protein was affected in this system, whence when the YDA crude sample was passed through the column was degraded afterwards. We used aprotinin and PMSF as protease inhibitors during all the expression and purification steps but only two smaller fragments than YDA were found enriched in the elution fractions and recognized by Western blot.

Ammonium sulfate precipitation was also used to enrich the 96 kDa band which corresponds to full-length YDA and which appears in low concentrations in the sample. Saturation with 40% allowed us to elute this protein but also the 2 smaller proteins. Presently, purification at this step is still being implemented.

We decided to use agroinfiltration of *N. benthamiana* as an alternanative system for protein expression and purification. This system has the advantage of be a plant system and present similar conditions for the native protein. 5 or 6 days after agroinfiltration, GFP, which was fused to the C-end of YDA in the vector used for agroinfiltration, was successfully detected, but after protein extraction of all agroinfiltrated leaves the protein could not be detected. This supports the notion that YDA is rather unstable, even in a plant system. Kim *et al.* (2012) performed co-infiltration of BIN2 and YDA to perform overlay assays and evaluate protein-protein interactions. They harvested all the leaves after 36 hours and precipitated using YFP inmunoprecipitation.

They also used other protease inhibitors as bestatin, pepstatin and leupeptin. For *E. coli* the codon use change of YDA from Arabidopsis could allow its correct expression, stability and purification, and in the case of ZYMV the fragments samples could be precipitated also with ammonium sulfate of TCA, however the precipitation could affect the structure of the protein and therefore its function.

6.5   Vascular genes evolved from non-vascular ancestors, which were involved in other developmental pathways

YDA promoters and other genes that can be involved in vascular formation or function present specific motifs that could coordinate their expression in this tissue (Ruiz-Medrano *et al.,* 2011).

An interesting problem in evolution is how genes for functions essential in complex multicellular organisms arise. Several scenarios can be envisaged; for example, these genes could have homologs in simpler relatives that have another function, but have been recruited to fulfill a novel activity. Likewise, novel genes and even pathways could arise de novo, although this is not well documented. The case of the vascular tissue in plants is a good example of an evolutionary innovation that likely resulted from emergence of novel genes and recruitment of preexisting ones.

To analyze this with more detail, we selected genes that are required for vascular tissue formation and function, and more specifically in the case of phloem, for long-distance signaling. These genes were *APL, OPS, FT* and *CmPP16. APL* is a gene that is found in all members of viridiplantae, including chlorophytes. Its known function is related to phloem differentiation, but it also plays a role in flower induction as a transcriptional activator of *FT* and as part of the transport complex FTIP1 (Bonke *et al*., 2003; Abe *et al*., 2015). The presence of FT in non-vascular plants as well as in chlorophytes, i.e., likely before the emergence of vasculature and flowers, indicates that it is involved in other processes, possibly transcriptional activation during development. Long distance transport of FT is obviously a novel function that could be related to its transport to nucleus in more basal lineages in plants, as in the case of KNOTTED1 and other non-cell autonomous proteins (Lucas and Lee, 2004). Interestingly, FT homologs were found only in *C. subellipsoidea* in chlorophytes. Also, TCTP homologs were found only in this

species in chlorophytes (Martínez-Navarro *et al*., 2013; Toscano-Morales *et al*., 2015). No important duplication events appear to have occurred during evolution of FT in flowering plants. In dicots two different clades can be observed, and one of which is highly conserved between the members of the group.

FT and APL function could have converged after the emergence of flowers. It will be of interest, to determine how the interaction of these two proteins emerged, and whether this occurs in *C. subellipsoidea*. OPS determines phloem continuity; it has a different evolutionary pattern compared to FT and APL (Truernit *et al.,* 2012). It is only present in tracheophytes. The function of this protein appears to be exclusive of vascular plants, and its function seems to be related only to this tissue, although how it developed a regulatory function for BIN2 is still unclear (Anne *et al.*, 2015). CmPP16 was found to have different phylogenetic relationships. This protein is able to bind RNA in a non-specific manner, and transport it cell-to-cell through plasmodesmata and long-distance through the phloem, although its function is not clear yet. Its mRNA also moves long-distance through the phloem. Because of these activities this protein is considered as a paralog of viral movement proteins (Xoconostle-Cázares *et al.,* 1999). This protein is similar to the C-domain of synaptotagmin, involved in vesicle trafficking; more recently it has been found to be involved in the movement of a plant virus to the cell periphery, which could be related to the potential role of CmPP16 in cell-to-cell transport of RNA (Schapire *et al.,* 2009, Lewis *et al.,* 2010). It should be noted that the CmPP16 phylogeny does not reflect the most accepted grouping of viridiplantae (as mentioned before, the pumpkin CmPP16 is more related *to S. moellendorffii* than to other dicot proteins). This suggests that this gene has unequal substitution

rates between different clades, and/or that there has been lateral gene transfer, at least in the case of members of this gene family.
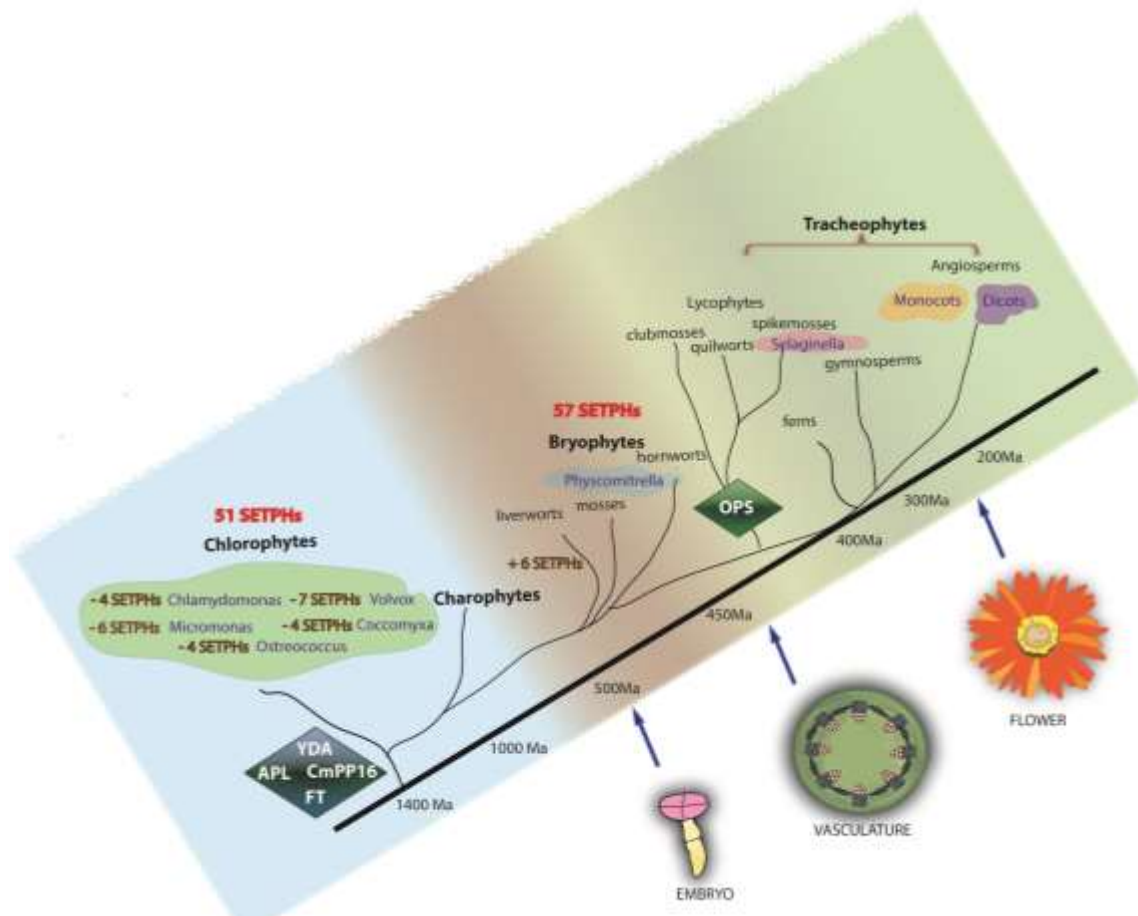
Figure 38. Scheme of sieve element transcript promoters homologs (SETPHs). YDA, APL, CmPP16 and FT are ancient genes that acquired novel and specific functions in the function and development of the vasculature. OPS is a new gene appearing after emergence of the vasculature. In red is shown the number of SETPHs that were found also in taxa lacking vascular tissue. Image Martínez-Navarro *et al.*, 2013

The MAPK protein family is much larger than in mammals and other organisms. The phylogenetic analysis of YDA shows an ancient origin of this gene. YDA has numerous homologs in both bryophytes and tracheophytes, possibly by duplication events. In cucumber YDA harbors two homologs that could have originated from a duplication event and then each one specialized for a certain function, in contrast with Arabidopsis YDA. According to the full-length protein tree, YDA homologs are present also in chlorophytes; it could be speculated that in the ancestor of

the land plant ancestor it have been involved in regulating processes such as colonization or multicelullarity. YDA can be distinguished from other MAPK members of its group (MAPK Group 2002, Figure 18) by two particular regions, the N-terminal and C-terminal domains. Sopeña–Torres (2015) suggests that the N-terminal domain could be the key region for stress response regulation, particularly in plant immunity. There are no data to relate the C-terminal domain with a specific function or expression pattern of YDA in plants; however, analysis with MatGat performed in this work showed that reported YDA homologs share high percentage of identity and similarity with these, in contrast to other MAPKKKs from Arabidopsis (Figure 19). In *Carica papaya* the YDA homolog appears more related to chlorophytes than with other dicots. This could be due to the length of YDA in this organism, rather than be a real phylogenetic relationship.
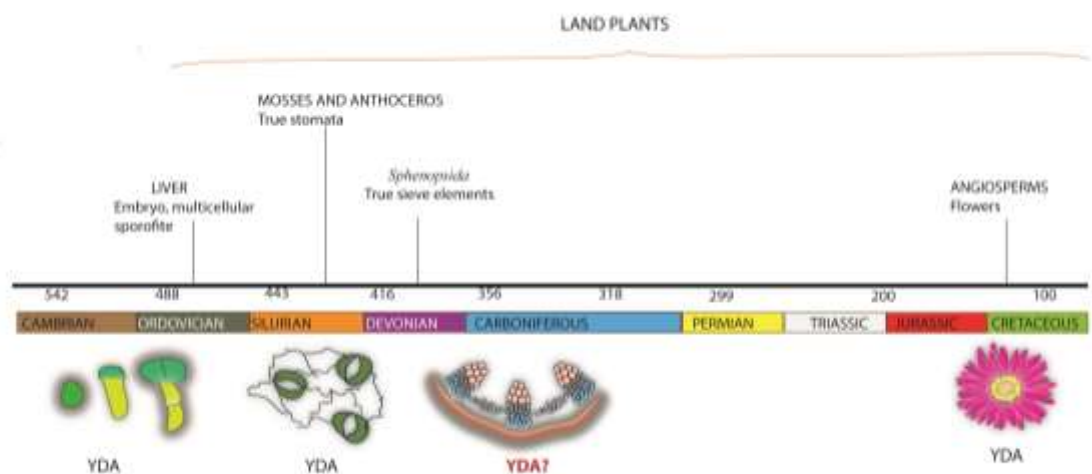
Figure 39. Evolution of YDA function. YDA was initially involved only in embryo development, however many elements of the YDA pathway are also involved in development of other tissues, indicating that there was a pathway recruitment during the evolution of the vasculature in plants.

Current evidence shows that *YDA* is a gene that participates both in development and response to certain stimuli. The expression of *YDA* in vascular tissue suggests that it has an active role in the development of this tissue. Additionally, upstream and downstream elements of the YDA pathway are shared by stomata, embryo, inflorescence and root developmental programs, as well as in other pathways. These tissues precede the origin of the vascular tissue; therefore we

propose that YDA and other proteins that were previously specialized in other processes could have been recruited for the formation of vascular tissue.

6.6 Possible relation of YDA MAPKKK in vascular tissue development with the ERECTA pathway

Vascular tissue development involves the formation of xylem and phloem. Xylem has been extensively studied, and it is known that some genes involved in auxin response have an important role in this process (Figure 39). The *HD Zip III* and *KANADI* gene families are also reported to determine the abaxial and adaxial patterning in leaves. Other genes such as VND are also known to participate in formation of vessels, and fibers of xylem. In phloem only the proteins that participate before phloem differentiation and involved in stem cell maintenance have been described. Functioning in a manner similar to Xylogen, OPS determines the phloem cell lineage while APL is more directly related to formation of sieve elements and companion cell. The pathways involved in the control of differentiation of mature phloem, of which OPS and APL form part are not clear yet.

YDA has a role in vascular tissue formation, possibly acting downstream of ERECTA receptors and upstream of MKK4/5 and MAPK3/6. The MAPK function could be related to BIN2 regulation as has been reported in stomata, and controlled by OPS in protophloem differentiation (Figure 39). Based on the *YDA* expression pattern it is currently not clear if it is acting in cambium cells and whether its function is more related to stem cell maintenance or cell differentiation. Further analysis will help determine the underlying mechanism of the YDA cascade in vascular tissue development. The specific constructs in which the *YDA* ORF is fused to the *SUC2, IRX3* and *APL* promoters could help to determine which tissues and cells are affected by its loss of function. This could be further explored using complementation assays with the *YDA* mutants analyzed in this work.
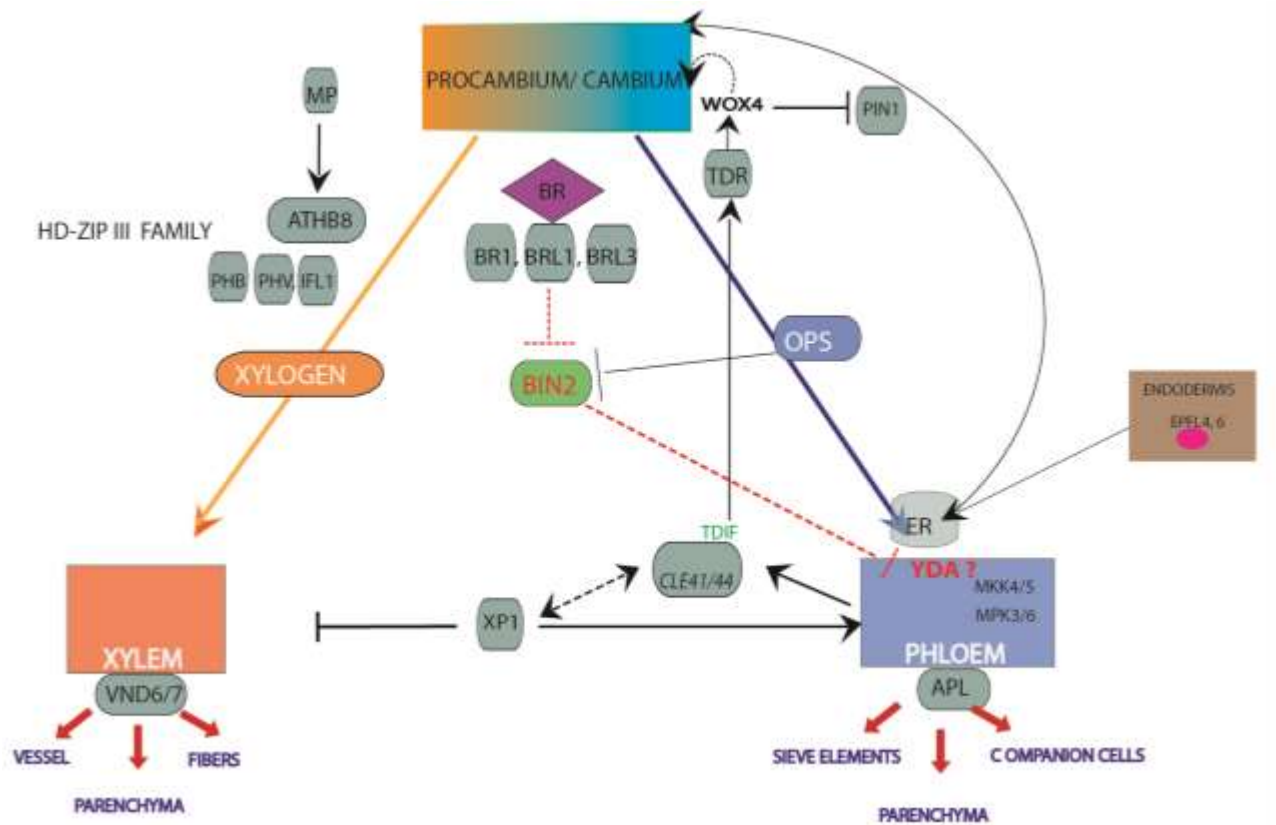
Figure 40. Model for vascular tissue development. Procambium/cambium cells give rise to xylem and phloem. Patterning genes as well as hormone-regulated pathways help determine vasculature cell lineages. Many genes act in stem cell maintenance. YDA is depicted in phloem cells downstream of the ERECTA receptor, although the exact function of these genes is still unclear.

CONCLUSIONS AND PERSPECTIVES

7 CONCLUSIONS AND PERSPECTIVES

7.1 Conclusions

In previous work in our group it was observed that *YDA* is expressed in the vasculature, including the phloem. This gene has an important role regulating asymmetric cell division in the embryo, as well as during stomatal differentiation. Given that phloem differentiation involves asymmetric cell division, which gives rise to the mature CC-SE complex and thus the functional phloem, we hypothesized that this gene could also have a role in vascular tissue differentiation. Below are some conclusions derived from the present work.

- We found that *YDA* is expressed in all plant tissues during the adult stage. Expression pattern varies in rosette and cauline leaves. *YDA* is found in xylem and phloem cells in inflorescence stem and carpels of mature flowers.

- Protein localization analysis of YDA plants harboring a YDA-GFP construct driven by the *35S* promoter shows that the protein accumulates in vascular tissue of inflorescence stem.

- The phenotype of the *yda-1* and *emb71* mutants shows germination and growth delay. They also exhibit alteration in stem shape as well as in vascular bundle patterning causing flattening and decreased number of cambium and phloem cells compared to WT plants.

- *YDA* is transcriptionally regulated by different growth regulators during seedling and adult stages. Cytokinin and SA repressed the expression of *YDA* in seedlings while ABA, GA, SA, BR and auxin repressed its expression in leaves, stem and apex in adult plants.

- UBQ10 is not a good normalizer gene for growth regulators treatments given that it is induced in adult plants by some of them such as SA, and auxins

- The two homologs of YDA in cucumber are highly similar, and are both highly expressed in vascular bundles; thus, it is probable that they have redundant functions, and they may have originated from a gene duplication event.

- *APL, FT, CmPP16* and *YDA* are ancient genes that could have been recruited during evolution from an ancestor lacking vasculature, and acquired novel functions involved in vascular tissue development and physiology.

- OPS is not found in non-vascular plants, suggesting that it is a novel gene exclusive of tracheophytes and specialized in vascular tissue development.

7.2   PERSPECTIVES

The almost ubiquitous localization of YDA and the phenotype of mutants showed that it is an important factor not only in stomata and embryogenesis but also in vasculature development in inflorescence stem of Arabidopsis. Further analyses will be necessary to elucidate with detail the mechanisms underlying the role of YDA MAPK pathway in vascular tissue development. Complementation assays as well as analysis of the phenotype of plants overexpressing *YDA* crossed with *YDA* mutants are key experiments to determine the exact role of this protein in the vasculature. The *SUC2, IRX3* and *APL* promoters will help to determine which cell type is altered as a result of *YDA* loss of function. A specific promoter for cambium cells, such as WOX4, could be also used for this assay. Likewise, it will be of interest to rescue the phenotype of *ER* and *OPS* mutants by overexpression of YDA using the aforementioned tissue specific promoters.

Protein-protein interaction assays in this tissue will help to determine which factors are downstream and upstream of YDA and are required for vascular tissue development. Specific assays should be performed also for the three domains of YDA, since it has been reported that the N-terminal domain is likely involved in plant immunity.

SUPPLEMENTARY INFORMATION

8 SUPPLEMENTARY INFORMATION

Table S1. Takara reaction mix

Ex taq Buffer 10X	1.25 μl
DNTP's 2.5 mM	1 μl
Primer 5' (10 mm)	0.25μl
Primer 3' (10 mm)	0.25μl
TAKARA	0.05μl
DNA (30-50 ng)	1μl
H$_2$0	8.2 μl
Total reaction volume	12.5 μl

Table S2. TOPO ligation mix

PCR product	2 μl
Salt solution	0.5μl
PCR8GWTOPO	0.5μl
Total reaction volume	3 μl

Table S 3 LR clonase II reaction

Entry Vector (~200 ng)	1 μl
Destination Vector (450 ng)	1 μl
LR Clonase II	0.5μl
TE Buffer	2.5 μl
Total reaction volume	5 μl

Table S4 Primers and conditions used for sequence amplification

PRIMER	SEQUENCE 5'-3'	AMPLIFICATION PROGRAM
YDA promoter	Forward: TTGATCAAATCATGCAATTAGAAAGA Reverse: ATCTTCTTCCCACAAACCAAGAGGCA	94°C 3 min 94°C 35 sec 58°C 35 sec 35 cycles 72°C 1.5 min 72°C 5 min
pYda-YDA	Forward: TTGATCAAATCATGCAATTAGAAAGA Reverse: GGGTCCTCTGTTTGTTGATCCGGGAGT	94°C 3 min 94°C 35 sec 58°C 35 sec 35 cycles 72°C 4.5 min 72°C 5 min
ORF YDA	Forward: ATGCCTTGGTGGAGTAAATCAAAAGAT Reverse: GGGTCCTCTGTTTGTTGATCCGGGAGT	94°C 3 min 94°C 35 sec 62°C 35 sec 35 cycles 72°C 2.5 min 72°C 5 min
COOH YDA	Forward: GGAGTAGTCCAAGGACTCCTCCTCATG Reverse: GGGTCCTCTGTTTGTTGATCCGGGAGT	94°C 3 min 94°C 35 sec 60°C 35 sec 35 cycles 72°C 2.5 min 72°C 5 min
SUC2 promoter	Forward: ACCTACATTTATAAATAAAATCTGGTT Reverse: TTTGACAAACCAAGAAAGTAAGAAAAAA	94°C 3 min 94°C 35 sec 52°C 35 sec 35 cycles 72°C 2.5 min 72°C 5 min
APL promoter	Forward: AGTTATGTTTCAAAATAGCGTTAGATT Reverse: TCTCTCTCTCTCTCTCTGACTCTCTTTCTCTCTCTGAAAGAG	94°C 3 min 94°C 35 sec 54°C 35 sec 35 cycles

| | | 72°C 2.5 min |
| | | 72°C 5 min |

| IRX3 promoter | Forward: CCTATTGGTAGTTCTGCGAAACGT Reverse: AGGGACGGCCGGAGATTAGCA | 94°C 3 min 94°C 35 sec 61°C 35 sec 35 cycles 72°C 2.5 min 72°C 5 min |

Table S 5 GFP primers and amplification conditions

PRIMER	SEQUENCE 5'-3'	AMPLIFICATION PROGRAM
GFP ORF	Forward: ATGGTGAGCAAGGGCGAGGAGCTG Reverse: CCTTGTACAGCTCGTCCATGC	94°C 3 min 94°C 35 sec 65°C 35 sec 35 cycles 72°C 40 sec 72°C 5 min
BAR ORF	Forward: ATGAGCCCAGAACGACGCCCGGCCG Reverse: AAATCTCGGTGACGGGCAGGACCGG	94°C 3 min 94°C 35 sec 62°C 35 sec 35 cycles 72°C 40 sec 72°C 5 min
P35S COOH	Forward: TGAGACTTTTCAACAAAGGATAATTT Reverse: GGGTCCTCTGTTTGTTGATCCGGGAGT	94°C 3 min 94°C 35 sec 52°C 35 sec 35 cycles 72°C 40 sec 72°C 5 min

Table S6. Restriction enzyme reaction mix

Cut Smart Buffer	2 µl
DNA (250 ng -1µg)	1- 4 µl
XbaI	0.4 µl
H20	Up to 20 µl
NEB Buffer 3.1	2 µl
DNA (250 ng -1µg)	1- 4 µl
HincII	0.4 µl
H20	Up to 20 µl

NEB Buffer 3.1	2 µl
DNA (250 ng -1µg)	1- 4 µl
EcoRV	0.4 µl
H20	Up to 20 µl
NEB Buffer EcoRI	2 µl
DNA (250 ng -1µg)	1- 4 µl
EcoRi	0.4 µl
H20	Up to 20 µl

Table S7. Concentration of growth regulators

GROWTH REGULATOR	CONCENTRATION
AUXIN (IAA)	1, 10 μM
CYTOKININ (Zeatin)	1, 10 μM
ABSICIC ACID	1, 10 μM
GIBERRELIC ACID (GA$_3$)	1, 10 μM
SALICYLIC ACID	100 nM, 1 μM
BRASSINOSTEROID (Brassinolide)	100 nM, 1 μM

Table S8. Sequence of primers used for relative quantification of transcripts.

PRIMER	SEQUENCE 5´- 3´	KAPA Amplification program
YDA RTPCR	Forward: CAAGGACTCCTCCTCATGTATTT	
	Reverse: GGACACATGATCAGATAGGACTG	42°C 5 min 95°C 3 min
UBC10	Forward: GATCTTTGCCGGAAAACAATTGGAGGATGGT	95°C 3 sec 40 cycles 60°C 20 sec
	Reverse:CGACTTGTCATTAGAAAGAAAGAGATAACAGG	60- 95°C 1°C/1min

Table S9. Primers for amplification of *Cucumis sativus YDA* homologs.

PRIMER	SEQUENCE 5´- 3´	KAPA Amplification program
Cucsa107950	Forward: CTCACATCTTCTCTGAAATGATACCA Reverse: AATACCATTCATGCGACCCGACAAT	
Cucsa129470.	Forward: TTCAGCCAGGCTCACACATCTTTTC Reverse: TTTGCGGTTGGTCAAAAGAGAGCTTG	42°C 5 min 95°C 3 min 95°C 3 sec 40 cycles
ACTIN 8	Forward: CCATGACGGGATCACATTTC Reverse: CAAACGCTGTAACCGGAAAG	60°C 20 sec 60- 95°C 1°C/1min

Table S10. Phusion PCR reaction and ligation

Phire buffer 5X	2.5 μl
DNTP's 10 mM	0.25 μl
Primer 5' (10 mm)	0.625μl
Primer 3' (10 mm)	0625 μl
Phire hot start II	0.25 μl
DNA (30-50 ng)	1 μl
H_2O	7.25 μl
Total reaction volume	12.5 μl

Table S11. Primers for amplification and cloning in ZYMV vector.

PRIMER	SEQUENCE 5'-3'	AMPLIFICATION PROGRAM
N-terminal	Forward: GCATGCATGCATGCCTTGGTGGAGTAAATCAA Reverse: GGTAGGTACCCGATTCAGGGCTAACCGTAGCC	98°C 30 sec 98°C 5 sec 55°C 5 sec 35 cycles 72°C 30 sec 72°C 1 min
MAPK domain	Forward: GCATGCATGCATGCGATGGAAAAAAGGGAGAT Reverse: GGTAGGTACCTACAAAAGCATGATCCAAAAGC	98°C 30 sec 98°C 5 sec 55°C 5 sec 35 cycles 72°C 30 sec 72°C 1 min
C-terminal	Forward: GCATGCATGCATGAGAAATGTGATGCCGATGG Reverse: GGTAGGTACCGGGTCCTCTGTTTGTTGATCCG	98°C 30 sec 98°C 5 sec 55°C 5 sec 35 cycles 72°C 30 sec 72°C 1 min

Table S12. Ligation reaction and Restriction enzymes mix

5X Rapid Buffer	2.5 μl	NEB Buffer 1.1	2 μl
Lineal vector	0.25 μl	DNA (250 ng -1μg)	1- 4 μl
DNA Fragment	0.625μl	SphI	0.4 μl
T4 ligase (ThermoScientific)	0625 μl	KpnI	0.4 μl
Total volume	0.25 μl	H20	Up to 20 μl

Table S13. pTrcHis2 ligation

PCR product	2.5 µl
Ptrchis2	0.5µl
Total volume	3µl

Table S14. Western Blot Protocol

•	
• **Stacking gel 4%, Resolving gel 10-12%**	10% for YDA 96Kd and 12% for Cter, MAPK and GFP
• **Transfer:**	1hr 100 volts or overnight 30 volts
• **Blocking**:	Skim Milk 5% in TBS 1X (NaCl, Tris-HCl pH8) during 2 hours.
• **First antibody:** •	c-Myc (1:5000) and GFP(1:5000) in Skim milk 5% in TBS 1X during 2 hours
• **Washes**: •	3 with TBS 1 X during 10 minutes
• **Second antibody:**	IgG-HRP (1:5000) in Skim milk 5% in TBS 1X during 3 hours
• **Washes**:	5 with TBS 1 X during 10 minutes
• **Revealed**:	750 µl of solution A, 750 ml of solution B of ECL-GE Healthcare™ mixed and spreaded over the membrane during 3 minutes. Film exposition : 2- 4 minutes using Hyperfilm ECL-GE Healthcare™

Table S 15 Basal nutrient Solution. Taken from Conn *et al.* 2012

Basal Nutrient Solution					
Macronutrients	**FW**	**g to make 1 L stock**	**Stock Conc (M)**	**Vol of stock (mL) for 1L**	**Final conc (mM)**
NH_4NO_3	80	80	1	**2**	2
KNO_3	101.1	101.1	1	**3**	3
$CaCl_2$	1M solution		1	**0.1**	0.1
KCl	74.55	74.55	1	**2**	2
$Ca(NO_3)_2 \cdot 4H_2O$	236.1	94.4	0.4	**5**	2
$MgSO_4 \cdot 7H_2O$	246.5	98.6	0.4	**5**	2
KH_2PO_4	136.1	13.61	0.1	**6**	0.6
NaCl	58.44	58.44	1	**1.5**	1.5

Micronutrients	**FW**	**g to make 1 L stock**	**Stock Conc (mM)**	**Vol of stock (mL) for 1L**	**Final conc (uM)**
NaFe(III)EDTA	367.1	18.4	50	**1**	50
H_3BO_3	61.8	3.09	50	**1**	50
$MnCl_2 \cdot 4H_2O$	197.9	0.99	5	**1**	5
$ZnSO_4 \cdot 7H_2O$	287.5	2.875	10	**1**	10
$CuSO_4 \cdot 5H_2O$	249.7	0.125	0.5	**1**	0.5
Na_2MoO_3	242	0.0245	0.1	**1**	0.1

pH with NaOH to 5.6	

MACRONUTRIENTS

MICRONUTRIENTS

Final Conc of i	mM	Activity	Final Conc of i	mM	Activity
K	5.6	4.79	Fe	0.01	25 pM
Ca	2.1	1.05	Mn	0.005	23nM
Mg	2	1.03	Zn	0.01	50 uM
NH_4	2	1.72	Cu	0.0005	23 nM
Cl	3.71	3.19	Mo	0.0001	31 nM

NO$_3$	9	7.75
SO$_4$	2.0105	0.893
PO$_4$	0.6	1.8 pM
Na	1.5502	1.38

Table S 16 Terrific broth (TB)

Solution A 650ml	Solution B 100 ml
Tryptone 8.67 g	KH$_2$PO$_4$ 1.73 g
Yeast extract 17.3 g	K$_2$HPO$_4$ 9.4 g
Glycerol 2.88 g	pH 7

Table S 17 LB medium for bacteria

LB medium	1L
Tryptone	5 g
Yeast extract	2.5 g
NaCl	5 g
Agar 5g/L just for solid medium	

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9 REFERENCES

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