ESTABLISHING TRANSGENIC *ARABIDOPSIS* PLANTS FOR PROTEIN FUNCTION AND POLAR DISTRIBUTION ANALYSIS

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ABSTRACT

An important aspect of cell biology is the asymmetric or polar distribution of proteins within the cell. Proteins that have polar distribution have important functions in plant growth and development, however, few plant proteins with polar localization, excluding transporters, have been identified. This project was designed to initiate transgenic *Arabidopsis thaliana* plants for protein function and polar distribution analysis. Two transmembrane receptor kinases, WALLFLOWER (WFL) and KINASE ON THE INSIDE (KOIN), are localized to the inner side of the plasma membrane in root cells. To understand how these proteins localize we generated truncated versions that are missing the intracellular kinase domain. The truncated versions of each protein failed to show its normal polar localization. Thus, we hypothesized that specific regions of the kinase domain are important for WFL and KOIN localization and function. To test this, specific amino acids were targeted for mutation and then mutant versions of each of these proteins were tagged with fluorescent protein; then, integrated into plant expression vectors. The expression vectors were validated to confirm that the specific mutations were present and then the expression vectors were transformed into *Arabidopsis* plants. Seeds from the first generation after transformation were collected, grown on medium containing a selective agent, then transplanted to grow to maturity. Later generations of these plants will be analyzed for protein localization and function.

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INTRODUCTION

The roots of plants are vital structures that primarily keep plants anchored to the ground and provide a large surface area for plants to absorb water and mineral nutrients, such as phosphorus, nitrogen, and potassium, from the soil. In addition, they serve as centers of storage, conduction, hormone synthesis, colonial regeneration, and rhizospheric interactions. Plant root research has been extensively conducted since 1980s. Roots are now used to study soil remediation, nutrient cycling, genetics, tissue growth patterns and modelling, and other emerging fields (Lux et al, 2012).

Much like how the mouse is the premier mammalian model organism for biomedical research, *Arabidopsis thaliana* is an ideal model organism for plant biology because it is easy to maintain and grow in the lab. It is a small plant, often considered a weed that self-reproduces and has a short life cycle of ~8 weeks with one plant yielding many seeds. Studies on this relatively simple angiosperm have produced significant advances in understanding plant growth and development (Meinke et al, 1998). Root growth and development consists of cell division, elongation, and differentiation or cellular specialization. These processes are regulated by plant hormones, genetic factors, and various environmental conditions, including water availability, temperature, light intensity, nutrient availability, and biotic factors.

Arabidopsis root tip consists of three developmental zones, the meristematic, transition, and elongation zones (Fig. 1A). An important feature in the meristematic zone is a stem cell niche, which contains more frequently dividing initial cells that will give rise to all the different root cell types and the quiescence center. The quiescence center (QC) contains a largely non-dividing pool of stem cells inside of the root apical meristem (RAM) and maintains dividing initial cells in the QC surrounding. The initial cells will form all tissues that are essential for root function after a

few cycles of cell division and differentiation. Thus, QC is very crucial for root growth and development. However, its molecular mechanism and intertwined regulatory network are under investigation. It may require more complex control by a combination of symmetric and asymmetric division (Strotmann et al, 2021).

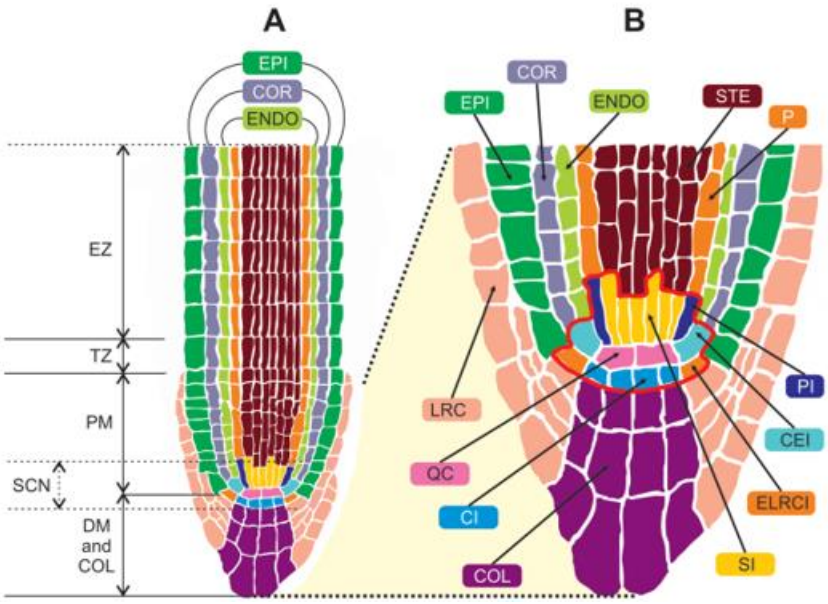


Figure 1. Structure of the root (Lee et al, 2013)

The past few decades have witnessed various advancements and innovations in the field of plant molecular biology. Various bacterial and plant genes involved in tumorigenesis were discovered. Agrobacterium, which mediates transformation in plants, was discovered and very quickly become adopted as the most popular plant transformation vector. This bacterium can be used to transform plants with genes of interest by replacing the naturally occurring oncogenes endogenous to the agrobacteria with the engineered gene of interest. Unlike the previous methods that required tissue culture, agrobacterium transformation was based on incubating mature seeds with *agrobacterium*, growing plants from these treated seeds, and then screening for antibiotic resistance among the progeny seedlings (Lee et al, 2013).

The advantages of Agrobacterium-mediated transformation are being able to integrate pieces of DNA with defined ends and minimal rearrangement, which will ensure the high quality and fertility of the transgenic plant. The relatively small, genetically tractable genome of Arabidopsis thaliana makes genome manipulation via genetic engineering more easily and quickly.

Single-cell pollen tubes or root hairs of Arabidopsis are used as model systems to study the molecular and cellular biological and genetic bases of plant cell polarity (Yang et al, 2008). Cell polarity is essential for biological processes in eukaryotic growth and development and is particularly important in plant biology. Cell polarity refers to the asymmetric distribution of cellular components, including receptor kinases in the plasma membrane (Van Norman, 2020; Van Norman 2016). In *Arabidopsis thaliana* there are ~220 transmembrane receptor kinases or so-called Leucine-Rich Repeats Receptor-Like Kinase (LRR-RLK) (Shiu et al, 2001). These transmembrane receptor kinases likely function to perceive signals from other cells and are members of a large gene family in plants that are mainly involved in developmental and stress processes. The LRR containing domain of these proteins is on the extracellular side and kinase domain is on the intracellular side. These two domains are connected by a transmembrane domain.

The identification of the two *Arabidopsis* LRR-RLKs called WALLFLOWER (WFL) and KINASE ON THE INSIDE (KOIN) in the Van Norman lab provides a new set of conceptual and molecular tools to investigate cell polarity in plants. Both kinases have polar localization in root cells. WFL gene is polarly localized to the inner plasma membrane domain in the epidermis and the lateral root cap (Toth et al, 2022). WFL protein within epidermal hair cells have polar localization in the elongation and differentiation zones of the root. It is concentration at the inner domain of epidermal hair cells. Additionally, after deleting the intracellular domains, WFL's

asymmetric distribution is re-localized to the outer domain of the cells, which is opposite of the un-truncated protein (Toth et al, 2022). KOIN, the second LRR-RLK, limits the divisions of cells in the root meristem of Arabidopsis. In the endodermis, KOIN localizes to the inner polar domain of the endodermis and in the stele. KOIN's extracellular domains are insufficient for polar sorting and function. (Rodriguez-Furlan et al, 2022). KOIN function to regulate cell division to maintain overall meristem size (Rodriguez-Furlan et al, 2022).

Previous experiments reported by the Van Norman lab indicate that truncated versions of WFL and KOIN, which are missing the intracellular kinase domain, WLFΔK and KOINΔK respectively, do not localize properly. Specifically, WFLΔK localizes to the outer polar domain of root cells (Toth et al, 2022) and KOINΔK (Rodriguez-Furlan et al, 2022) shows nonpolar (uniform) localization in the plasma membrane of root cells. These results indicate that the kinase domain of each protein is important for its proper localization.

WFL and KOIN are 39.7% identical and 55.5% similar at the amino acid level with their kinase domains being 65% similar (Van Norman, 2020). WFL and KOIN kinase domains are predicted to be inactive or "atypical" as they contain the motifs to bind ATP but exhibit non-conserved residues at key positions necessary for phosphate transfer (Van Norman, 2020). To assess the function of these kinase domains, we introduced point mutations in the ATP binding domains. Additionally, we created versions that introduce the conserved residues from typical kinases into WFL and KOIN to create versions that more closely resemble a typical, active kinase. For each of these, transgenic mutant plants expressing the mutant versions fused to GFP will be generated to test how these mutant proteins localize and whether they carry out their normal functions *in planta* by assessing whether they can complement the *wfl* and *koin* null alleles, respectively.

OBJECTIVES

To build expression vectors containing mutated versions of the *wfl-1* and *koin* receptor kinases to be expressed in plants. These will be used as tools to determine whether specific residues of the kinase domain are important for *wfl-1* and *koin* localization and function.

MATERIALS AND METHODS

Plant and E. coli materials and growth conditions

Arabidopsis thaliana Col-0 was used as the wild type (WT) plant and for genomic DNA. Mutant versions of the kinase domains of WFL and KOIN were generated previously by site-directed mutagenesis and verified by DNA sequencing. Then these coding regions were fused with that of green fluorescent protein (GFP) and integrated into plant expression vectors. Expression vectors containing WFL(mut)-GFP and KOIN(mut)-GFP were transformed into chemically competent E. coli, transformed E. coli were selected on lysogeny broth (LB) Agar plates containing 60 ug/ml Spectinomycin. Then the plasmids were validated, catalogued, and stored, after which these vectors were used for plant transformation by standard approaches. Individual *Arabidopsis thaliana* transformants were selected and grown to the next generation for analysis. Seeds from the first generation after transformation were bulk collected, then surface sterilized by chlorine gas for 2 hours four times for T1 plants or once for all other plants. T1 plants are the first generation of plants with either WFL(mut)-GFP or KOIN(mut)-GFP point mutated genes. Sterilized seeds were then sown on a growth medium 1X MS (Murashige and Skoog), 1 % sucrose, 1% agar growth medium containing Norflurazon (Norf) or glufosinate ammonium (Basta) as selective agents and with 100 ug/ml Cefotaxime (Cef) to inhibit agrobacterum growth. The plates were then stratified at 4 °C in the dark for at least two overnights before moving to a Percival growth chamber set at 22˚C with 16 hours/8 hours light/dark for 5-10 days. Resistant T1 seedlings were moved from selection plates to 1X MS plates for recovery for 7 days. T1 plants were transplanted to soil and allowed to grow until senescence. Plants of subsequent generations (T2s and T3s) will be collected and analyzed for localization and function of WFL and KOIN.

koin-1 mutant plants lack the KOIN protein and *wfl-1* mutants lack the WFL protein. Expression of the mutant versions of these proteins in the mutant backgrounds can show whether the mutated protein restores normal protein function. Col-0 is the wild type of plant and will serve as a control for the mutant plants.

Vector construction and validation

Expression vectors containing WFL(mut)-GFP and KOIN(mut)-GFP were previously constructed using established protocols with Gateway © compatible reagents.

The *WFL* promotor (*pWFL)* is active in the lateral root cap and maturing epidermal cells, while the *SCR* promotor (*pSCR*) is active in the endodermis. The *KOIN* promotor (*pKOIN*) is active in the stele and endodermis of plant roots. Since these promoters have specific patterns of activity, the expression of the proteins driven by them are expected to localize in the promoter defined tissues regardless of genotype.

The expression vectors were extracted from transformed E. coli with in-house miniprep protocol [Appendix protocol #1], confirmed via restriction enzyme digestion using NotI and PvuII for WFL and NotI and EcoRI for KOIN, and then visualized with a 1% agarose gel run at 120 volts for 50 minutes. Each gel well contained 10 µL of DNA.

Agrobacterium transformation and colony PCR

Expression vectors containing WFL(mut)-GFP and KOIN(mut)-GFP were first transformed into agrobacterium by electroporation. Briefly, mixed well 0.5-1 ul of DNA (expression vectors) to 1 tube of electro competent Agrobacterial cells per transformation and conducted electroporation using the pre-programed Electro pulser in Yang/Reddy lab. Selected transformed agrobacteria by plating 8 dots on 60 ug/ml Spectinomycin + 20 ug/ml Gentamicin

(Gent) LB plates and incubated at 28˚C incubator for ~2 days or on benchtop for 3-4 days. Picked colonies and swirled mix with 35 ul dH_2O per colony then grow 3 hours to overnight on benchtop and took 2 µL of this culture for the PCR reaction. Transformed Agrobacteria were confirmed by PCR analysis according to the primer information. Primers should span the promoter to the gene of interest.

1) Forward primer: overlapping part of promoter and end of gene.

2) Reverse primer: overlapping exon to GFP.

Primers should allow amplification of 1.0-1.5 kb in length.

PCR reaction

	1x	5x	20x
Apex Red Taq	7.5 µL	37.5	150
Primer 1 (5 µM)	1.5 µL	7.5	30
Primer 2 (5 µM)	1.5 µL	7.5	30
Sterile H_2O	2.5 µL	12.5	50
DNA	2 µL		

*"DNA" was taken from cultures grown in water in PCR tubes

Set up PCR program

Annealing temperature based on melting temperature of primers

95˚C	3 min
95˚C	10 sec
56˚C	10 sec
72˚C	45 sec*
72˚C	3 min
4˚C	∞

35x

* Extension time varies based on length of fragment being amplified (30s per 1 kb)

Colonies with the construct of interest were grown up for use in transformation using a standard floral dip protocol. Briefly, 250 ml of agrobacterium culture was grown for 48 hours. The agrobacteria were separated by centrifugation, then they were re-suspended in a 250 mL dipping solution of 5% sugar and 100 µl of silwet. Silwet is a surfactant used to bypass the waxy cuticle

on plant leaves. Removed siliques if they have started to develop. The inflorescence stems of the plants were soaked in the dipping solution for a 1- 2 minutes, and then laid horizontally into a tray container lined with paper towels. The tray was then covered and placed in the dark overnight to allow recovery time. The next day the plants were placed upright.

koin-1 mutant plants lack the KOIN protein and *wfl-1* mutants lack the WFL protein. Expression of the mutant versions of these proteins in the mutant backgrounds can show whether or not the mutated protein restores normal protein function. Col-0 is an the wild type plant and will serve as a control for the mutant plants.

Seed Sterilization by chlorine gas

Seeds (3 ml max per tube) were surface sterilized by chlorine gas. In briefly, in a chemical fume hood, placed 3 ml maximum of seeds in 15 mL conical tubes in a rack in the dome next to a beaker with 100 ml of 100% bleach, then added about 3 ml 10N chlorine acid, keep tube cap loose, replaced dome lid, and closed hood. For Agro-dipped TIs, sterilized 3-4 times with fresh Bleach _HCL solution every 2 hours or once for all other plants.

Transplanting

After agrobacterium transformation, the plants were transferred to Sunshine potting mix soil to grow to maturity. Briefly, plants were gently removed from plate, transplanted 5 plants per pot, pots were covered with plastic wrap for 1-3 overnights. Plants were checked ~3x weekly for growth/watering.

RESULTS

Screening and transformation of expression vectors containing WFL(mut)-GFP

Expression vectors containing WFL(mut)-GFP fusions were assessed by restriction enzyme digestion with NotI with gel electrophoresis showing cleavage products of 6481 bp, 3014 bp, 2063 bp, and 314 bp and with PvuII showing cleavage products of 6481 bp, 4050 bp, 1992 bp, and 872 bp . For example, expression vectors driven by the SCARECROW promoter pSCR and containing WFL_A484F-GFP was examined (Fig. 2). The nomenclature for the vector construct name is promoter : gene_mutation : reporter. For example, in pSCR:WFL_G464R:GFP, pSCR is the SCARECROW promoter. WFL is the WFL gene. G464R is the induced mutation, which in this case is a single nucleotide polymorphism (SNP). GFP is the reporter protein. The mutation G464R means that amino acid G (glycine) at position 464 was replaced with amino acid R (arginine).

After confirmation of the expression vector, they were transformed in agrobacterium and then these colonies were tested by Polymerase Chain Reaction (PCR) to ensure the correct DNA fragment was present. Four WFL(mut)-GFP expression vectors were transformed into Agrobacterium, including pSCR:WFL_GN464-465RD:GFP, pSCR:WFL_G350E:GFP, pSCR:WFL_G464R:GFP, pSCR:WFL_A484F:GFP. An example of the PCR assay is shown in Fig. 3 with the pSCR:WFL_GN464-465RD:GFP expression vector.

Screening of the expression vectors containing KOIN(mut)-GFP by the same way as WFL(mut)-GFP above

The same screening process that was used for *wlf-1* expression vectors was also performed for KOIN-1 expression vectors. DNA restriction digestion with NotI and gel electrophoresis results

yield about 6484 bp, 3089 bp, 2063 bp, and 314 bp and with EcoR1 and get results yield 7559bp and 4391bp . Digestion analysis of pSCR:PLK4_GN484-485RD:GFP is shown in Fig. 4. In this gel, despite 314bp not showing up, pSCR:PLK4_GN484-485RD:GFP is still confirmed correct.

Agrobacterium transformation and Colony PCR were performed for four KOIN(mut)-GFP expression vectors as well. They were pSCR:KOIN-1_G484R:GFP, pKOIN-1:KOIN-1_G484R:GFP, pSCR:KOIN-1_GN484_485RD:GFP, pKOIN-1:KOIN-1_GN484_485RD:GFP. An example of the PCR assay is shown in Fig. 5 with the pKOIN-1:KOIN-1_G484R:GFP expression vector.

A summary of the vectors screened is shown in table 1 and 2 respectively, the numbers indicate how many E.coli or agrobacteria colonies for each vector passed each step of the screening. The "x" indicates that the step was performed.

In Table 1, nine out of fourteen WFL vectors were screened, five were not available. Three of nine were in T1 seeds that were previously derived from transformed *Arabidopsis* Col-1 or *wfl-1* plants, one was from an extracted DNA stock, the remaining five screened vectors were either in E coli stocks or needed to be constructed. T1 seeds of 3 vectors were selected for further function and polar distribution analysis.

pSCR: WFL_K369R:GFP was transformed into Col-0 and *wfl-1* plants. This yielded two successfully transformed Col-0 T1 plants and four *wfl-1* T1 plants. Eighteen E coli cultures were made to establish more transformed plants. Nine of sixteen E coli culture tubes yielded plasmid DNA extracted. However, none of the nine plasmid extracts was verified by the restriction enzyme digest analysis.

For pWFL: WFL_K369R:GFP, T1 seeds for both Col-0 plants and *wfl-1* plants were already available so they were grown directly on to transformed media. Two Col-0 plants and nine *wfl-1* plants were selected.

For pSCR:WFL_EA483-484DF:GFP, T1 seed for both Col-0 plants and *wfl-1* plants were also already available so they were grown directly on to transformed media. However n,o T1 plants from either Col-0 or *wfl-1* germinated.

DNA extracts of pSCR:WFL_GN464-465RD:GFP were available so they were directly transformed into Agrobacterium. Total of sixteen agrobacterium colonies were confirmed. One of the sixteen agrobacterium colonies was used to transform both Col-0 and wfl-1 plants. Four Col-0 and five wfl-1 T1 plants were selected.

pSCR:WFL_G350E:GFP had extracted DNA available for transformation into Agrobacterium. Total of sixteen agrobacterium colonies were confirmed. However, only five *wfl-1* T1 plants were selected. Two E coli cultures were created for Col-0 T1 plants. Both cultures have successfully extracted DNA but failed the digest verification.

pWFL:WFL_G350E:GFP T1 seeds for Col-0 plants and *wfl-1* plants were available to plate. 2 Col-0 and six *wfl-1* plants were selected.

pSCR:WFL_G464R:GFP has twelve successful extracts form twelve E coli culture tubes. Four of which were digest verified. 8 agrobacterium were PCR verified. Two Col-0 T1 plants and eight *wfl-1* T1 plants were selected.

pSCR:WFL_C67Y:GFP had eleven E coli tubes, but only six were successfully extracted. None was verified by digestion.

pSCR:WFL_A484F:GFP had eighteen E coli tube. One failed DNA extraction. Four passed the plasmid digestion. Eight Agrobacterium colonies were verified. However, none of the T1 plants was successful.

In Table 2, twelve of fourteen KOIN vectors were screened. Five of which needed *E. coli* culture to scale up. Three of which needed to be transformed into agrobacteria. Three of the screened vectors were screened for resistant T1 seedlings. Seven KOIN expressed in plants were selected to sue for further function and polar distribution analysis.

pSCR:KOIN_G484R:GFP and pKOIN: KOIN_G484R:GFP had extracted DNA available for agrobacterium transformation. Only one of pSCR:KOIN_G484R:GFP and nine of pKOIN: KOIN_G484R:GFP were verified by PCR. For pSCR:KOIN_G484R:GFP , only two Col-0 T1 plants were selected for.

pSCR: KOIN_D503N:GFP was transformed into plants. Four Col-0 and eight *koin-1* T1 plants was selected. Nine out of sixteen E coli tubes had DNA successfully extracted. However, none were verified by digestion.

Twelve of fifteen pKOIN: KOIN_D503N:GFP E coli tubes had DNA successfully extracted. None was confirmed correct by digestion.

pSCR: KOIN_C82Y:GFP was transformed into Col-0 and *koin-1* plants. Only fifteen *koin-1* T1 plants was selected. None of the Col-1 was selected.

pKOIN: KOIN_C82Y:GFP had only Col-0 seed available to plate on selection media for T1 selection. Unfortunately, none of the Col-0 plants was selected.

pKOIN: KOIN_C63Y:GFP was transformed into Col-0 and *koin-1* plants. Five Col-0 and twenty two *koin-1* T1 plants were selected.

pKOIN: KOIN_C63Y:GFP and pKOIN-1: KOIN-1_C63Y:GFP both were amplified in E coli culture. pKOIN: KOIN_C63Y:GFP had four culture tubes. All of pKOIN: KOIN_C63Y:GFP DNA were successfully extracted from the culture tubes. Ten pKOIN: KOIN_C63Y:GFP DNA in 10 E coli culture tubes were successfully extracted. None of either construct was verified by the digestion analysis.

pSCR: KOIN_GN484-485RD:GFP had two DNA extracts extracted successfully from two tubes of E coli culture. These two DNA extracts were verified by digestion. Only two agrobacterium was PCR confirmed to have the correct pSCR: KOIN_GN484-485RD:GFP plasmid. Only two Col-0 and one *koin-1* T1 plant were selected.

pKOIN: KOIN_GN484-485RD:GFP had extracted DNA available. However, none of the agrobacterium cultures was PCR verified.

Seven pSCR: KOIN_G67E:GFP DNA plasmids were extracted from seven E coli tubes. However, none of which was confirmed by digestion analysis.

Table 1. Summary of screening activities for WFL(mutant) vectors.

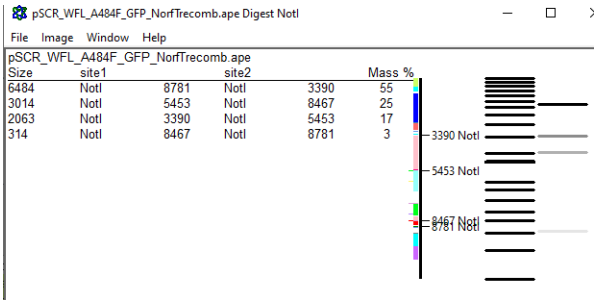
vector	E coli cultures tubes	Plasmid DNA extraction	Plasmid digest	Transform agro	PCR	transform plants	T1 Selection
pSCR:WFL_K369R:GFP	16	9	0			x	2 (Col-0), 4 (plk3-1)
pWFL:WFL_K369R:GFP							2 (Col-0), 9 (plk3-1)
pSCR:WFL_EA483-484DF:GFP							0 (Col-0), 0 (plk3-1)
pWFL:WFL_EA483-484DF:GFP							
pSCR:WFL_GN464-465RD:GFP				x	16	x	4 (Col-0), 5 (plk3-1)
pWFL:WFL_GN464-465RD:GFP							
pSCR:WFL_G350E:GFP	2	2	0	x	16	x	0 (Col-0), 5 (plk3-1)
pWFL:WFL_G350E:GFP							2 (Col-0), 6 (plk3-1)
pSCR:WFL_G464R:GFP	12	12	4	x	8	x	2 (Col-0), 8 (plk3-1)
pWFL:WFL_G464R:GFP							
pSCR:WFL_C67Y:GFP	11	6	0				
pWFL:WFL_C67Y:GFP							
pSCR:WFL_A484F:GFP	18	17	4	x	8	x	0 (Col-0), 0 (plk3-1)
pWFL:WFL_A484F:GFP							

Table *2*. Summary of screening activities for KOIN (mutant) vectors.

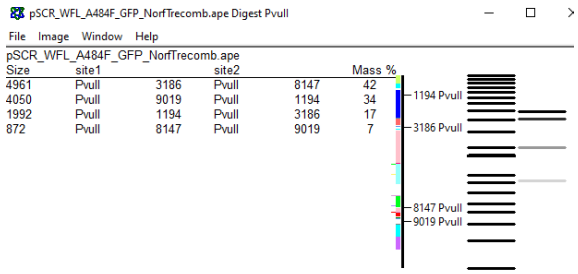
vector	E coli cultures tubes	Plasmid DNA extraction	Plasmid digest	Transform agro	PCR	transform plants	T1 Selection
pSCR:KOIN_G484R:GFP				x	1	x	2 (Col-0), 0 (WisDsLox439H07.9)
pKOIN: KOIN_G484R:GFP				x	9	x	3 (Col-0), 16 (WisDsLox439H07.9)
pSCR: KOIN_D503N:GFP	15	15	0			x	4 (Col-0), 8 (plk4-1.2.1)
pKOIN: KOIN_D503N:GFP	15	12	0				
pSCR: KOIN_C82Y:GFP						x	0 (Col-0), 15 (plk4-1)
pKOIN: KOIN_C82Y:GFP							0 (Col-0)
pSCR: KOIN_C63Y:GFP							
pKOIN: KOIN_C63Y:GFP						x	5 (Col-0), 22 (plk4-1)
pSCR: KOIN_K386R:GFP	4	4	0				
pKOIN: KOIN_K386R:GFP	10	10	0				
pSCR: KOIN_GN484-485RD:GFP	2	2	2	x	2	x	2 (Col-0), 1 (plk4-1)
pKOIN: KOIN_GN484-485RD:GFP				x	0		
pSCR: KOIN_G67E:GFP	7	7	0				
pKOIN: KOIN_G67E:GFP							

Confirming expression vectors containing WFL(mut)-GFP

a

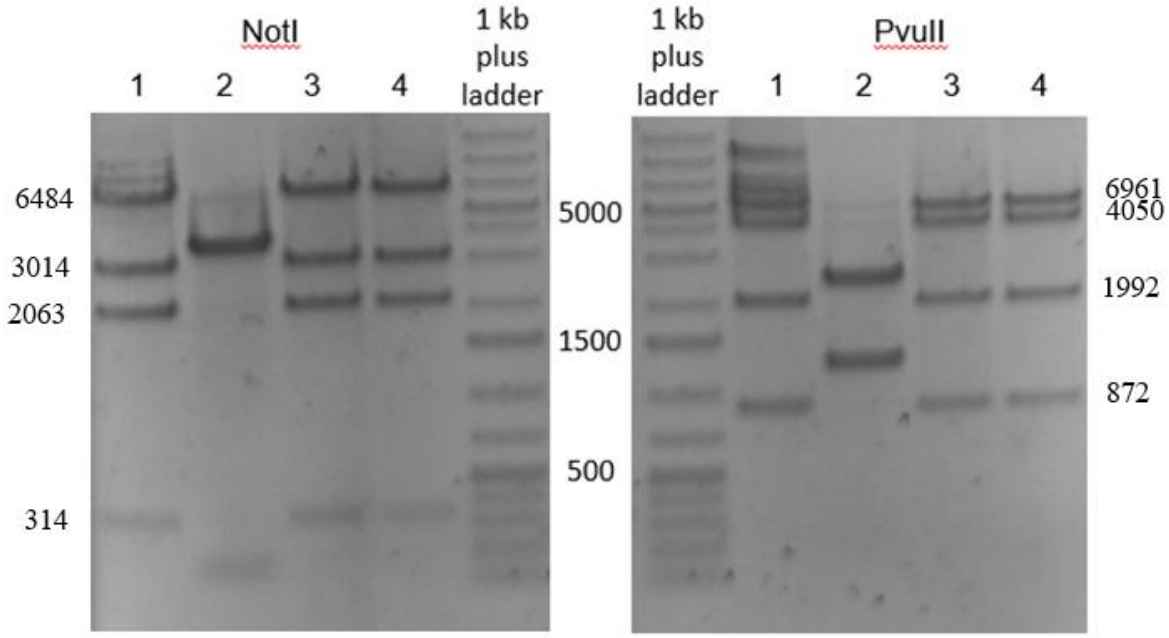


b



pSCR: WFL_A484F:GFP #1-4

c

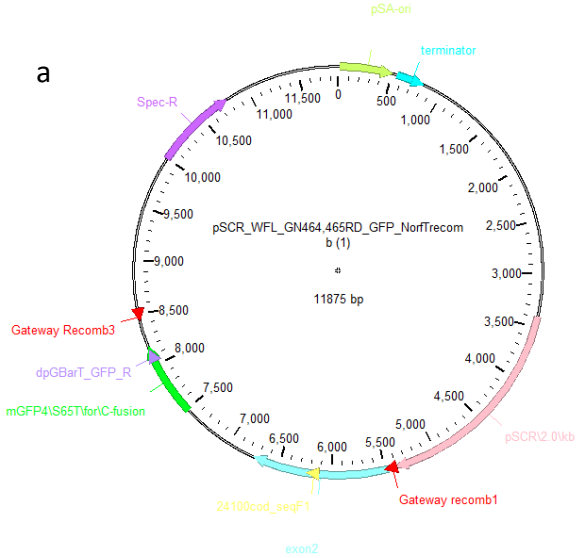


1% agarose gel run at 120mA for 50min

Figure 2. pSCR: WFL_A484F:GFP a) NotI digestion map, b) PvuII digestion map, c) NotI and PvuII digestion results

We used gel electrophoresis to illustrate the components of WFL mutant plasmid. pSCR_WFL_A484F_GFP plasmid was digested by *NotI* and *PvuII* restriction enzyme. The four bands are corresponding to the restriction map

Validation of agrobacterium-mediated transformation of WFL(mut)-GFP



Expression vector with PCR primers (24100cod_seqF1 (forward), dpGBarT_GFP_R (reverse).The length is 1936bp. This vector was used to integrate WFL mutant for function and localization analysis.

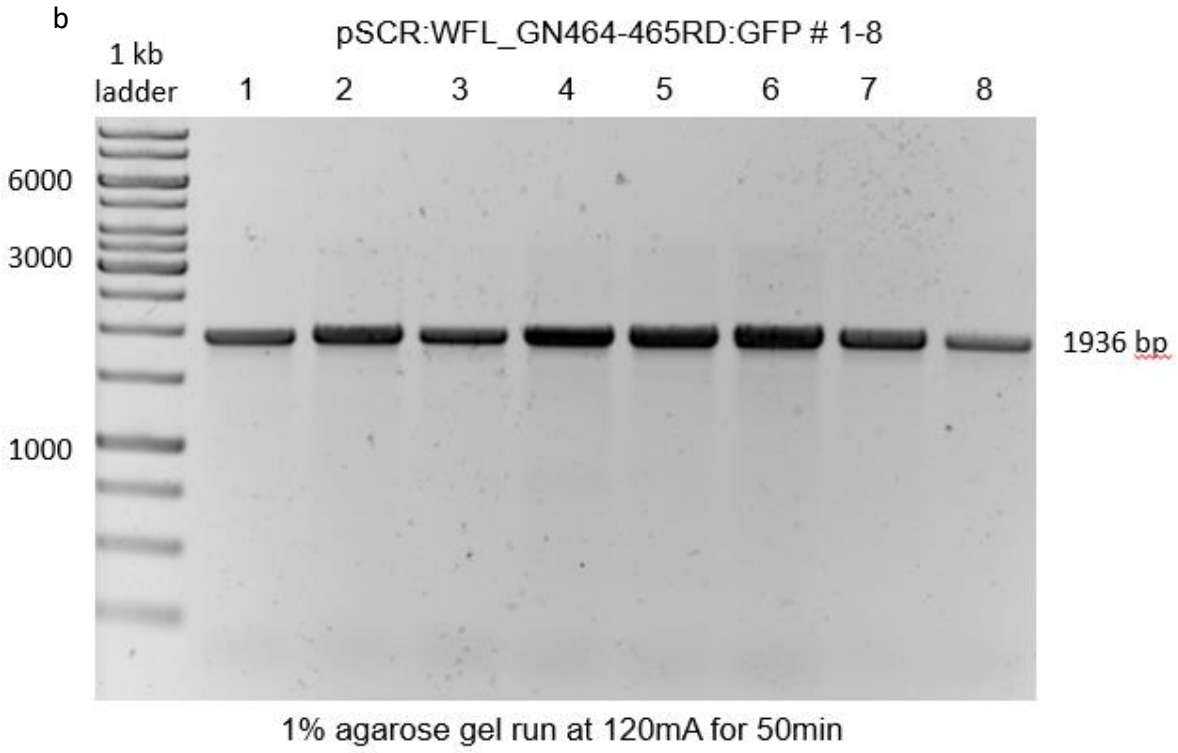


Figure 3. pSCR:WFL_GN464R-465RD:GFP a) expression vector, b) PCR result

We used gel electrophoresis to illustrate the PCR product (1936bp)

Confirming expression vectors containing KOIN(mut)-GFP

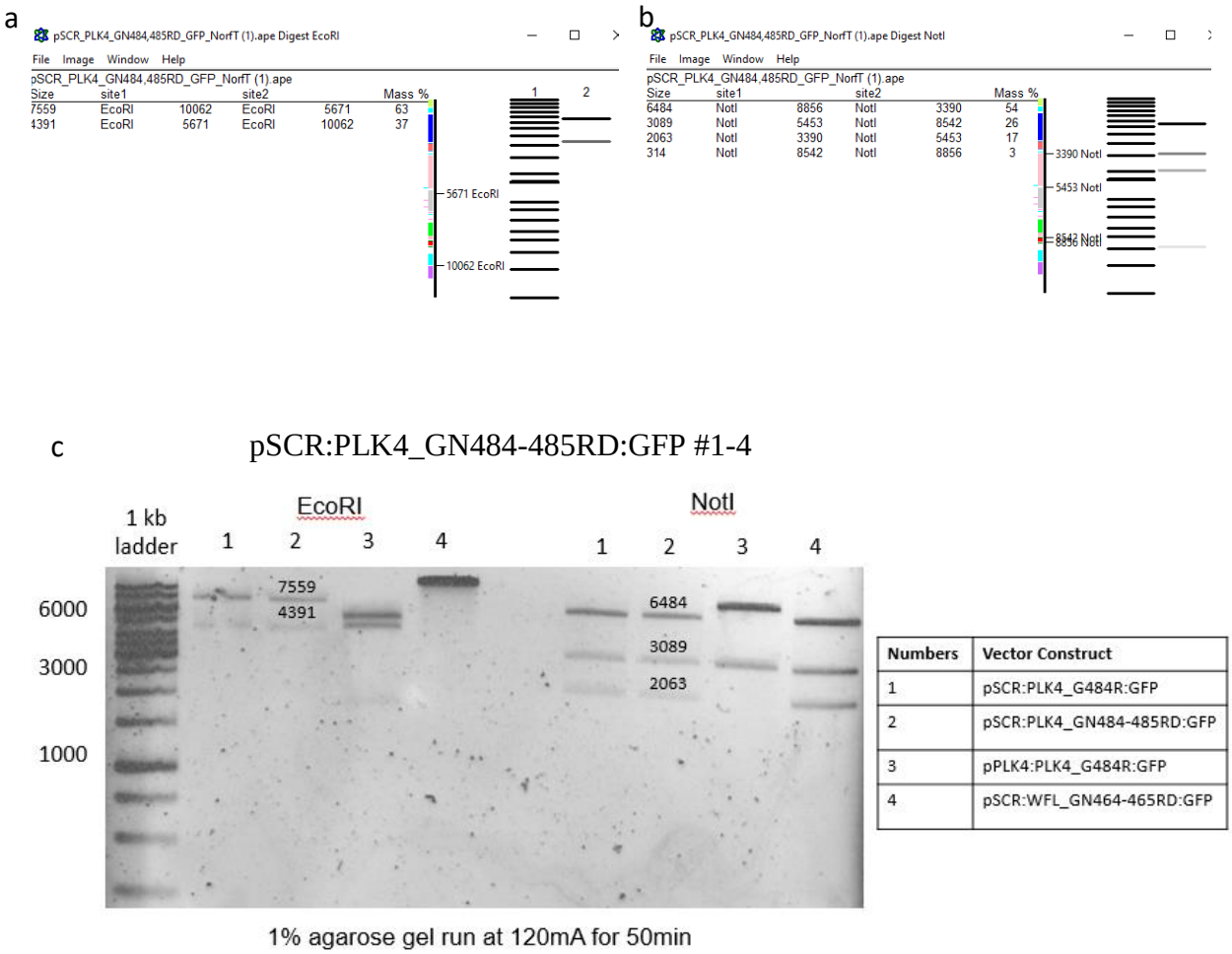
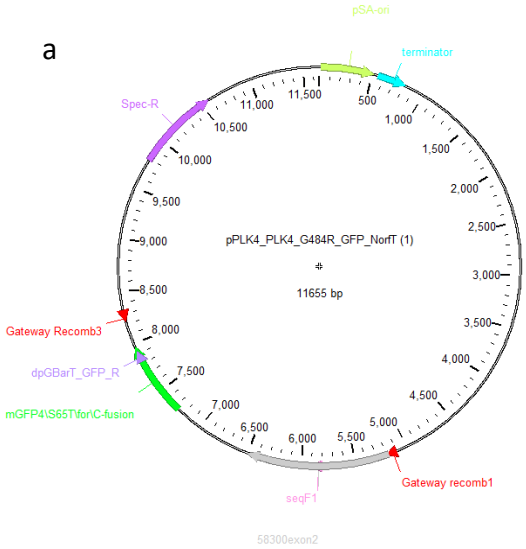


Figure 4. pSCR:PLK4_GN484-485RD:GFP a) EcoRI digestion map, b) NotI digestion map, c) EcoRI and NotI digestion results

We used gel electrophoresis to illustrate the components of KOIN mutant plasmid. *pSCR:PLK4_GN484-485RD:GFP* plasmid was digested by *EcoRI* and *NotI* restriction enzyme. The bands are corresponding to the restriction map except the 4th band at 314bp was invisible. The PLK4 is known as KOIN.

Validation of agrobacterium-mediated transformation of KOIN(mut)-GFP



Vector diagram showing the PCR primer sites (seqF1 (forward primer), dpGBarT_GFP_R (reverse primer). PCR amplicon length is ~2040bp. This vector was used to integrate KOIN mutant for function and localization analysis. PLK4 is also called KOIN.

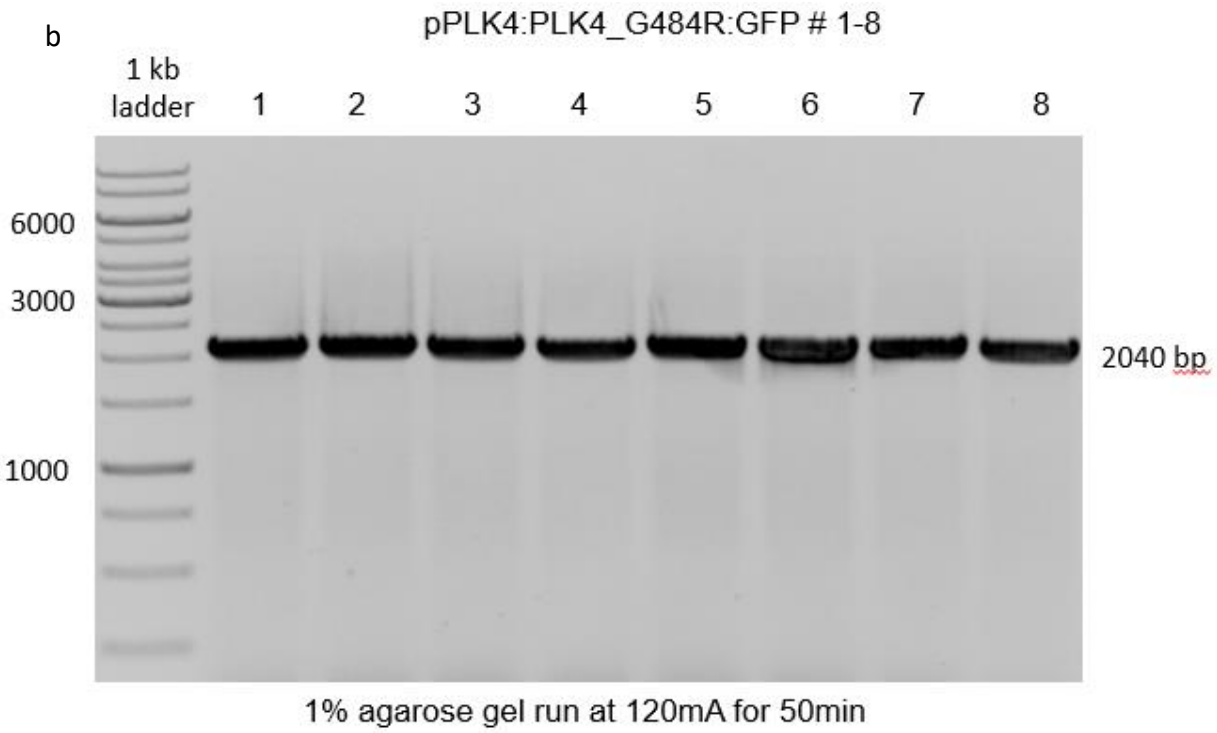


Figure 5. pPLK4:PLK4_G484R:GFP a) expression vector, b) PCR result

We used gel electrophoresis to illustrate the PCR product (2040bp), PLK4 is known as KOIN.

SUMMARY AND FUTURE DIRECTIONS

We generated the transgenic plants expressing mutant versions of WFL and KOIN to create tools to determine whether specific residues of the kinase domain are important for WFL and KOIN localization and function.

Transgenic plants expressing WFL and KOIN mutant versions were established. The T1 plants are the progeny of the plants that were transformed by agrobacterium. Only gametes of the transformed plants were actually transformed, then the fused egg cell fuses with a sperm cell to form a T1 seed. The resistant T1 plants are the 1st generation of plants that contain the expression vector. Their progeny (T2 plants) will be screened with 3:1 inheritance ratio and be analyzed with a confocal microscope for function and localization study.

Four out of seven WFL vectors were successfully transformed into both Col-0 and *wfl-1* plants as evidenced by resistant T1 progeny. The resistant T1 plants will proceed to 3:1 ratio T2 screening. Those vectors only transformed into *wfl-1* plants will need to be transformed to Col-0. Additionally, four out of the seven KOIN vectors were successfully transformed into both Col-0 and *koin-1* plants and will proceed to 3:1 ratio T2 screening. Two vectors that were transformed into either Col-0 or *koin-1* plants need to be re-transformation into either genotype, respectively, before the experiments can proceed.

Based on the previous experiments reported by the Van Norman lab, the truncated versions of WFL(Toth et al, 2022) and KOIN (Rodriguez-Furlan et al, 2022) proteins failed to show their normal polar localization (Fig. 7, Fig. 8b, and Fig. 9, right). The truncated version of WFL, missing the intracellular kinase domain is polarized to the opposite (outer) side depending on region of root

examined (Toth et al, 2022). While the KOIN extracellular domains are insufficient for its polar sorting and function in the endodermis (Rodriguez-Furlan et al, 2022). This suggests that there are different regions of these polarized receptors that are important for polar localization. Alternatively, it might also suggest that amino acids within the transmembrane domains of WFL and KOIN may be important for localization and function of these proteins. For example, if nonpolar Glycine (G) in the KOIN transmembrane domain is changed to positively charged Lysine (K), it might affect localization and be unable to properly function due to the amino acid change. However, if positively charged Lysine (K) is changed to a positively charged Arginine (R), it might remain polarly localized to the inner side of endodermis and be able to conduct its normal function (Fig. 6, Fig. 8a, and Fig. 9, left). This result would suggest that amino acid charge in the transmembrane domain is important for KOIN localization and function. The importance of the transmembrane domain in WFL and/or KOIN function could be investigated in the future.

CITED FIGURES

Localization of full length WFL under SCR promoter

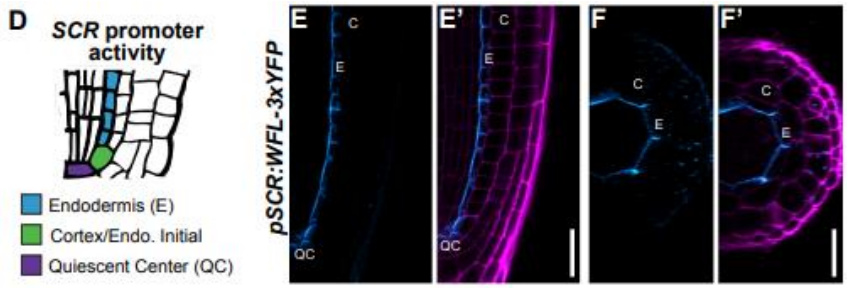


Figure 2. pPLK4:PLK4_G484R:GFP a) expression vector, b) PCR results

Localization of truncated WFL under SCR promoter in lateral roots

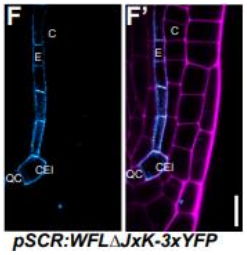


Figure 3. Localization of truncated WFL under SCR promoter in lateral roots (Toth, 2022)

Localization of truncated WFL under SCR promoter in root hair

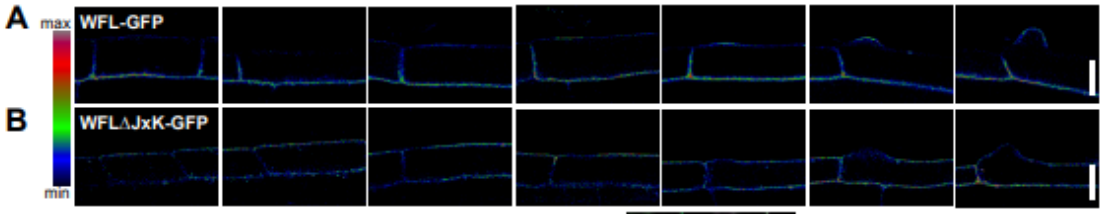


Figure 4. Localization of truncated WFL under SCR promoter in root hair (Toth, 2022)

Localization of full length and truncated KOIN under SCR promoter

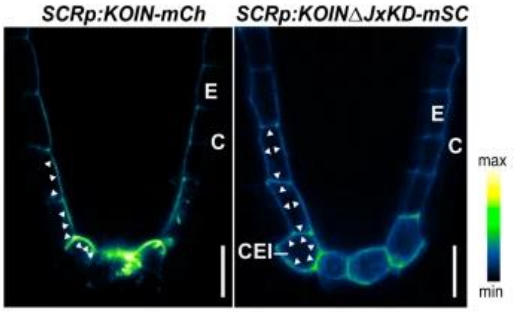


Figure 5. Localization of full length (left) and truncated (right) KOIN under SCR promoter (Rodriguez-Furlan, 2022)

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Appendix

Homemade Plasmid Mini Prep Protocol from Van Norman lab
Materials Needed:

1. Resuspension Buffer (Sol I)
2. Lysis Buffer (Sol II)
3. Neutralization Buffer (Sol III)
4. Isopropanol (cold)
5. 70% Ethanol (cold)
6. Overnight grown bacterial culture (1-5ml, typical 3ml)

Before you start:

1. Verify that lysis solution (Sol. II) is within its 7-day shelf life. Make a fresh solution, if it has expired.
2. Chill on ice resuspension (Sol. I), neutralization (Sol. III), isopropanol, and 70% ethanol solutions prior to starting
3. Generally, 1.5-3 mL of overnight culture is sufficient for extracting sufficient plasmid DNA for downstream applications.

Step	Procedure
1	Transfer 1.5mL overnight culture to a 1.7 mL microfuge tube.
2	Spin at 6000rpm for 5 minutes.
3	Decant LB medium from tube. For a higher concentration of DNA use 2-3 mL of overnight culture - note you will have to do 2 spins for this.
4	Add 200µL of cold resuspension buffer (Sol. I) to pellet
5	Resuspend pellet by vortexing
6	Incubate tubes on ice for 5 min
7	Add 200µL Lysis Solution (Sol. II) mix by inversion. Do not vortex.
8	Incubate on ice 3-5 min. Don't allow the lysis reaction to proceed longer than 5 min. (5 minutes starts when you add lysis buffer to tube 1.)
9	Add 150µL cold Neutralization buffer (Sol. III), mix by inversion
10	Centrifuge 13000rpm for 10min. If supernatant contains suspended particles repeat Step 10.
11	Transfer supernatant to new microfuge tube. Be careful NOT to transfer any precipitate. (Using a 200 µL pipet tip can help with this.)
12	Add 500µL cold isopropanol to the supernatant. Mix gently by inversion.
13	Spin 10-15min at 13000rpm. Decant supernatant.
14	Add 750µL cold 70% Ethanol. Mix gently by inversion.
15	Spin 10-15min at 13000rpm. Decant supernatant.
16	Air dry the pellet completely (either overnight (covered) or in a warm incubator as residual ethanol inhibits downstream applications).
17	Dissolve the pellet in 50-65µL 1X TE. The plasmid DNA is now ready for downstream applications or store at -20˚C for later use.