博士論文 （要約）

Analysis of gene function involved in plant organ development using *Arabidopsis thaliana* mutants.

シロイヌナズナ変異体を用いた植物器官形成に関わる遺伝子の解析

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Contents

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List of abbreviations

acl	*acaulis*
ACT8	*ACTIN8*
AG	*AGAMOUS*
AP3	*APETALA3*
ATML1	*ARABIDOPSIS THALIANA MERISTEM LAYER1*
BDG	*BODYGUARD*
CAPS	cleaved-amplified polymorphic sequence
CC	coiled-coil
Col	Columbia
EDS1	*ENHANCED DISEASE SUSCEPTIBILITY1*
ETI	Effector-triggered immunity
FDH	*FIDDELHEAD*
GA	gibberellin
GL2	*GLABRA2*
HD-ZIP IV	class IV homeodomain-leucine zipper
HDG	*HOMEODIMAIN GLABROUS*
HSP90	HEAT SHOCK PROTEIN90
KLU	KLUH
LFY	*LEAFY*
LTP	*LIPID TRANSFER PROTEIN*

L*er*	Landsberg *erecta*
MS	Murashige and Skoog
NBS-LRR	nucleotide binding site-leucine-rich repeat
NPR1	*NONEXPRESSOR OF PR GENES1*
OCL	*OUTER CELL LAYER*
PAD4	*PHYTOALEXIN-DEFICIENT4*
PCR	polymerase chain reaction
PDF1.2	*PLANT DEFENSIN1.2*
PDF2	*PROTODERMAL FACTOR2*
PI	*PISTILATA*
PR	pathogenesis-related
R	Resistance
RAR1	*REQUIRED FOR MLA12 RESISTANCE1*
RPP	recognition of *Peronospora parasitica*
RPT	regulatory particle triple-ATPase
RT	reverse transcriptase
SA	salicylic acid
SAD	START-adjacent
SAR	systemic acquired resistance
SEP3	*SEPALLATA3*
SGT1	SUPPRESSOR OF THE G2 ALLELE OF SKP1
SID	*SA INDUCTION DEFICIENT*

SNC1	SUPPRESSOR OF *NPR1*, CONSTITUTIVE1
SRFR1	SUPPRESSOR OF *rps4*-RLD
SSLP	simple sequence length polymorphism
START	steroidogenic acute regulatory protein-related lipid transfer
TIR	Toll/interleukin-1 receptor
TPR	tetratricopeptide repeat
UFO	*UNUSUAL FLORAL ORGANS*
ZLZ	zipper-loop-zipper

Abstract

Chapter I

acaulis1 (*acl1*) mutants are isolated in order to explore novel genetic factors that regulate elongation of inflorescence stem. In the first part, I report on the identification of an inversion mutation in the original *acl1-1* plants. Compared to the original *acl1-1* plants, the "genuine" *acl1-1* plants, which is without the inversion, grew larger and their inflorescence stems grew longer at 22°C and also at 24˚C. In the *acl1-1* plants with the inversion, two genes that locate at each end of the inversion were disrupted and full-length transcripts were not detected, and expressions of some genes within and adjacent to the inversion were also altered. These results suggest the possibility that the expression of multiple genes is involved in the enhancement of the *acl1-1* phenotype by the inversion .

In the second part, I further investigated the *acl1* mutants using the *acl1-1* mutant line without the inversion. I found Col accession-specific *Resistance* (*R*) gene, *SUPPRESSOR OF NPR1, CONSTITUTIVE1* (*SNC1*) as an essential gene for the *acl1* growth phenotype. Moreover, I identified the *acl1* mutations in *SUPPRESSOR OF rps4-RLD* (*SRFR1*), which is known as a negative regulator of defense responses and SNC1 activity. These results suggest that the loss of negative regulation of *SNC1* causes stunted growth in the *acl1* mutants. Consistent with the *srfr1* mutants, which have already been described, *acl1* mutants showed constitutive activation of defense-related genes at 22˚C. I further observed the *acl1* phenotype at intermediate temperatures

between 22˚C and 28˚C, at which the *srfr1* phenotype was reported to be suppressed. It was revealed that both stunted growth and increased expression of defense-related genes were gradually repressed as temperature increases and almost completely suppressed at temperatures above 26˚C. Double mutant analysis revealed that the *acl1* plant growth depends not only on *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) but also on *PHYTOALEXIN-DEFICIENT4* (*PAD4*) and *REQUIRED FOR MLA12 RESISTANCE1* (*RAR1*). However, salicylic acid accumulation and *NONEXPRESSOR OF PR GENES1* (*NPR1*)-dependent pathways were not essential for the *acl1* growth phenotype. I also discovered that higher ammonium concentration in the growth media alleviates the *acl1* phenotype. Nitrogen might be involved in the growth regulation of the plants with induced defense responses.

Chapter II

Development of the epidermis involves members of the class IV homeodomain-leucine zipper (HD-ZIP IV) transcription factors. The Arabidopsis HD-ZIP IV family consists of 16 members, among which *PROTODERMAL FACTOR2* (*PDF2*) and *ARABIDOPSIS THALIANA MERISTEM LAYER1* (*ATML1*) play an indispensable role in the differentiation of shoot epidermal cells. However, the functions of other HD-ZIP IV genes that are also expressed specifically in the shoot epidermis remain not fully elucidated. Construction of double mutant combinations of these HD-ZIP IV mutant alleles showed that the double mutants of *pdf2-1* with *homeodomain glabrous1-1* (*hdg1-1*), *hdg2-3, hdg5-1* and *hdg12-2,* produced abnormal flowers with

sepaloid petals and carpelloid stamens in association with reduced expression of the petal and stamen identity gene *APETALA3* (*AP3*). Expression of another petal and stamen identity gene *PISTILATA* (*PI*) was less affected in these mutants. I confirmed that the *AP3* expression in *pdf2-1 hdg2-3* was normally induced at initial stages of flower development but attenuated both in the epidermis and internal cell layers of developing flowers. Since the expression of *PDF2* and these HD-ZIP IV genes during floral organ formation is exclusively limited to the epidermal cell layer, these double mutations may have non-cell-autonomous effects on the *AP3* expression in the internal cell layers. My results suggest that cooperative functions of *PDF2* and other members of the HD-ZIP IV family in the epidermis are crucial for normal development of floral organs in Arabidopsis.

Cited and revised from Summary of Kamata *et al*., 2013. "Mutations in epidermis-specific HD-ZIP IV genes affect floral organ identity in *Arabidopsis thaliana*" *Plant Journal*, 75, 430-440.

General Introduction

Arabidopsis thaliana is a model plant that widely used for plant genome analysis (Somerville and Koornneef, 2002). Since a large collection of mutants and transgenic plants, whose growth and development have been disrupted, is available, a forward genetic approach has been practical for discovering genetic factors that regulate morphogenesis in *A. thaliana*. In the year 2000, the whole genome of the *A. thaliana* has been sequenced, enabling to presume the function of the genes that are not yet experimentally verified. Thus, the reverse genetic approach is also being a powerful tool to search and investigate whether and how the gene in concern is related to the plant growth and development.

Most organogenesis in plants, as well as in *A. thaliana*, is occurred postembryonically, unlike animals, in which the most of the organs are formed during embryogenesis (Carles and Fletcher, 2003). Both shoot and root apical meristems of plants maintain their activity and give rise to new organs after the germination, allowing plants to continue growing and developing their bodies through their lifetime. Although the postembryonic development of plant body is primarily genetically regulated, it is also highly flexible to the environmental factors, including biotic factors such as heat, cold, light, drought and nutrient condition, and biotic factors like attacks from pests and pathogens, to adapt to the environmental changes. The effects from the environmental changes on the plant growth and development cannot be ignored, as it has been suggested that environmental stresses can reduce average yields by as much as >50% for most major crop plants (Wang *et al*., 2003).

acaulis1 (*acl1*) mutants, which were previously isolated in a genetic screen for defective plant morphology, were further discovered to be an unique type of mutants that their growth phenotype is fully suppressed at higher growth temperatures (Tsukaya *et al*., 1993). Even though the growth of Arabidopsis plants is affected by higher temperature, the morphological modification in the *acl1* plants is so drastic that it cannot be explained by general effects from higher growth temperature (Thingnaes *et al*., 2003). Thus I thought that *acl1* would be more than a tool for investigating a genetic factor that regulate normal plant growth, and give us some more insight into a relationship between a growth regulatory pathway and the environmental factors, such as temperature. And in the first chapter of this thesis, I will report on the analysis on the *acl1* mutants. Since the *ACL1* gene had not been isolated, the biggest issue on the *acl1* analysis was to identify the *ACL1* gene, and to make it clear which genetic pathways are involved in the *acl1* plant growth. I identified some genetic factors that affect the *acl1* phenotype, and eventually identified the *acl1* mutations in a negative regulator of defense responses against pathogens.

As shown in the research on the *acl1* mutants, and also in some other *acl1*-like mutants (Gou and Hua, 2012), responses against pathogen attacks can dramatically modify the plant morphology. When plants are exposed to such pathogens, epidermis, the outermost cell layer that covers the plant body, plays the critical roles for the defense and resistance. Shoot epidermis is also important for organ separation and defense responses against drought or other environmental stresses as well as in the integrity of organs. Moreover, it has been reported that epidermis-specific genes are

involved in the regulation of organ development in plants (Savaldi-Goldstein *et al*., 2007; Eriksson *et al*., 2010). Therefore, it is likely that epidermis is an important cell layer for regulating both responses against environmental factors and plant growth. In the second chapter, I adopted reverse genetic approach to investigate the effects of T-DNA insertion mutations in the class IV homeodomain-leucine zipper (HD-ZIP IV) gene family, most of which are confirmed to be specifically expressed in epidermis (Nakamura *et al*., 2006), on plant growth and development.

Chapter I. Stunted growth of *acaulis1* (*acl1*) mutants is dependent on constitutive activation of defense response pathways.

Introduction

For exploring genetic factors that are essential for proper morphogenesis of *Arabidopsis thaliana,* a forward genetic approach has been a useful tool. Tsukaya *et al*. (1993) previously screened for mutants defective in the elongation of the inflorescence stem and able to identify five complementary groups of mutants, termed *acaulis* (*acl*), for their "stalkless" morphology. Short inflorescence stems and reduced number of flowers in the *acl* mutants were due to early proliferative arrest of apical inflorescence meristems. The number of rosette leaves in the *acl* mutants is approximately the same as in wild-type plants, indicating that the timing of transition from vegetative to reproductive phase is not affected. However, except for the *acl5* mutant, the *acl* mutants exhibited more or fewer defects in leaf morphology (Tsukaya *et al*., 1993; Hanzawa *et al*., 1997; Akamatsu *et al*., 1999). Since the *acl* phenotype cannot be rescued by the exogenous addition of several growth regulators and phytohormones, the stunted growth of the *acl1* mutants is considered to be different from the dwarfism of known phytohormone-related mutants (Tsukaya *et al*., 1993; Hanzawa *et al*., 1997; Akamatsu *et al*., 1999). The only *ACL* gene identified was *ACL5,* which encodes thermospermine synthase (Hanzawa *et al*., 2000; Kakehi *et al*., 2008). However, it is still uncertain which genetic pathway is involved in the regulation of plant growth and development of

other *acl* mutants.

acl1-1 is the most severely stunted mutant among the *acl* mutants. Cell elongation and maturation are likely to be inhibited soon after the cells differentiate in the *acl1-1* plants, as implied by the drastic reduction of cell length in the *acl1-1* stems and the loss of intercellular spaces in the *acl1-1* leaves (Tsukaya *et al*., 1993). In order to determine the role of the *ACL1* gene within the developmental network of inflorescences, double mutants were generated between the *acl1-1* mutant and some developmental mutations that affect the morphology of inflorescences and/or flowers. However, the function of the *ACL1* gene has been shown to be genetically independent of the shoot- and inflorescence-development genes, such as *APETALA1* (*AP1*), *CLAVATA* (*CLV1*), *LEAFY* (*LFY*) and *TERMINAL FLOWER1* (*TFL1*) (Tsukaya *et al*., 1993).

It has also been reported by Tsukaya *et al*. (1993) that stunted growth of the *acl1* mutants is restored at higher temperature, such as 28˚C. Ambient temperature is known to influence aspects of the appearances of plants, such as leaf size and stem length (Thingnaes *et al*., 2003; Atkin *et al*., 2006). However, the dramatic alteration in morphology of the *acl1* phenotype at high temperature cannot be explained by general developmental variations controlled by ambient temperature. Temperature-sensitive stunted phenotypes similar to the *acl1* mutants are often observed in *suppressor of NPR1, constitutive1-1* (*snc1-1*), *suppressor of npr1-5-based salicylic acid insensitivity4* (*ssi4*), *bonzai1* (*bon1*) and *constitutive expresser of PR genes 30* (*cpr30*), all of which have constitutive activation of defense responses against pathogens (Hua *et al*., 2001;

Shirano *et al.,* 2002; Yang and Hua, 2004; Gou *et al.,* 2009). Effector-triggered immunity (ETI) is a major defense response in plants that is induced by direct or indirect recognition of pathogen avirulence effectors by plant resistance (R) proteins (Jones and Dangl 2006; Alcazár and Parker 2011). In *Arabidopsis*, a majority of R proteins possess a nuclotide binding site and leucine-rich repeat (NBS-LRR) motif either with a Toll/interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain at the N terminus (Dangl and Jones, 2001). For the activation of downstream defense response pathways, TIR-NBS-LRR type R proteins require *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and *PHYTOALEXIN-DEFICIENT4* (*PAD4*) genes, both of which encode a protein with homology to lipases/acyl hydrolases (Falk *et al.,* 1999; Jirage *et al.,* 1999; Feys *et al.,* 2001). On the other hand, CC-NBS-LRR type R proteins require *NON RACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*), which encodes a putative membrane-bound protein (Century *et al.,* 1995; 1997; Aarts *et al.,* 1998). ETI is usually accompanied by a hypersensitive response involving rapid and local programmed cell death, which restricts further spread of the pathogen. Furthermore, plants produce salicylic acid (SA) and accumulate defense molecules such as pathogenesis-related (PR) proteins through systemic acquired resistance (SAR). SAR provides the plants with long-lasting protection against a broad spectrum of pathogens (Ryals *et al.,* 1996; van Loon, 1997).

The cloning of the *ACL1* gene will give us the answer how the *acl1* plant growth is regulated. During molecular mapping of the *acl1* mutations, I identified an inversion mutation in the original *acl1-1* mutant line, and a Col accession-specific

TIR-NBS-LRR *R* gene, *SUPPRESSOR OF NPR1, CONSTITUTIVE1* (*SNC1*) as an indispensable gene for the *acl1* phenotype to be exhibited in the presence of the *acl1* mutations. I eventually identified the *acl1* mutations in *SUPPRESSOR OF rps4-RLD* (*SRFR1*), a negative regulator of defense responses, suggesting that the *acl1* phenotype is caused by a constitutive activation of defense response pathways. I further analyzed the *acl1* mutants and will report on several phenotypes that yet have not been described in previous studies in the *acl1*/*srfr1* mutants.

Results

Part 1

An inversion identified in *acl1-1* mutant functions as an enhancer of the *acl1-1* phenotype

Identification of an inversion mutation in the original *acl1-1* mutant and isolation of novel *acl1-1* line without the inversion

Previous research showed that the *acl1-1* mutation locates close to *AGAMOUS* (*AG*) on chromosome 4 (Tsukaya *et al.*, 1993). My early results from molecular mapping suggested a linkage of the *acl1-1* phenotype between a cleaved-amplified polymorphic sequence (CAPS) marker, SC5, and the polymorphism MASC04642 on chromosome 4 (Figure I-1a). The *acl1-1* mutant was induced by X-ray irradiation and exhibits a more severe phenotype than the EMS-mutagenized *acl1-3*

mutant. Thus, I suspected that the chromosomes of the *acl1-1* mutant might be seriously disrupted. In order to explore chromosome disruptions, approximately 260 kb of the genomic region from *At4g22290*, the gene nearest to MASC04642, to *At4g21690* was examined by amplifying fragments covering the genomic regions by PCR. As the result, two PCR products including *At4g21960*, which encodes a peroxidase (Apel and Hirt, 2004; Welinder *et al*., 2002), and *At4g22250*, which encodes a zinc finger protein (Kosarev *et al*., 2002), were absent in the *acl1-1* mutant (Figures I-1a,b). The genomic regions between these two genes, which are located at a distance of approximately 120 kb, were found to exist in the *acl1-1* mutant (data not shown). Every PCR product was present in the *acl1-3* mutant, which indicated that the absence of the two PCR products was specific to the *acl1-1* mutant. One possibility was that the genomic region between *At4g21960* and *At4g22250* was inverted. To investigate this possibility, PCR experiments were performed using combinations of primers at *At4g21960* and *At4g22250*. Novel DNA fragments were amplified in the *acl1-1* mutant and their sequences suggested that *At4g21960* and *At4g22250* were cleaved, inverted and fused each other (Figure I-1c,d). I also cloned *At4g21960* and *At4g22250* from the *acl1-3* plants and found that there was no mutation. Since the T-DNA insertion lines in *At4g21960* and *At4g22250* had no obvious growth defects (data not shown), the loss of either of *At4g21960* or *At4g22250* is unlikely to be responsible for the *acl1-1* phenotype.

I crossed the original *acl1-1* plants to Col-0 to evaluate the segregation of the *acl1-1* phenotype and the inversion. The F2 population was segregated into 496 (73%)

wild-type plants and 180 (27%) *acl1-1* plants at a 3:1 ratio ($\chi^2 = 0.716$). I further examined 371 wild-type plants and 146 *acl1-1* plants for the inversion (Table I-1). Regarding the inversion, the segregation at +/+: *inv*/+: *inv*/*inv* at the ratio of 1:2:1 was less reliable (χ^2 0.025 (2) = 7.38 < χ^2 = 9.07 < χ^2 0.010 (2) = 9.21). The *acl1-1* phenotype did not necessarily cosegregate with the inversion, and the inversion is independent of the cause of the *acl1-1* phenotype. The recombination rate between the *acl1-1* phenotype and the inversion was estimated to be 15.4% according to the segregation of the F2 plants. To simplify descriptions, I refer to the newly isolated *acl1-1* plants without the inversion as *acl1-1* +/+, the original *acl1-1* plants with the inversion as *acl1-1 inv*/*inv*, wild-type plants as Col +/+, and the wild-type phenotype (*ACL1*/*ACL1*) plants with the inversion as Col *inv*/*inv*.

Expressions of genes related to the inversion

Inversions can disrupt a gene at one of its breakpoints, and furthermore, it is expected that inversions alter the expression of a gene near a breakpoint because of a change in its chromosomal environment. Expression of some genes, which locate within and adjacent to the inversion, differed among the four plant strains: Col +/+, Col *inv*/*inv,* *acl1-1* +/+, and *acl1-1 inv*/*inv* (Figure I-2a). I observed a decrease in expression of *At4g22270* (*IMMUTANS*) (Wu e*t al.,* 1999), an increase in *At4g22214* (defensin like protein) (Silverstein *et al*., 2005), and a slight increase in *At4g22235* (defensin like protein) in two *inv*/*inv* plants, Col *inv*/*inv* and *acl1-1 inv*/*inv*. In the *acl1-1 inv*/*inv* plants, we found increased expression of *At4g22050* (aspartyl protease family protein) and

At4g22070 (*WRKY DNA-BINDING PROTEIN 31*) (Eulgem *et al*., 2000) and decreased expression of *At4g21990* (*APS REDUCTASE 3*) (Houston *et al*., 2005), *At4g22010* (*SKU5 SIMILAR 4*) (Sedbrook *et al*., 2002) and *At4g22212* (defensin like protein). Among the genes investigated, *At4g22080* (pectate lyase family protein), *At4g22090* (pectate lyase family protein), *At4g22210* (Cys-rich protein), *At4g22217* (defensin like protein) and *At4g22230* (defensin like protein) decreased in both the *acl1-1* plants, regardless of the inversion. The expression of *At4g22030* (F-box family protein) and *At4g22100* (glycosyl hydrolase family 1 protein) was decreased in the Col *inv*/*inv* plants and the *acl1-1* +/+ plants, and even further decreased in the *acl1-1 inv*/*inv* plants compared to the Col +/+ plants. I wondered if the increase or the decrease in the expression level of these genes is due to mutations in their genomic sequences. However, the genomic sequences of these genes cloned from *acl1-1* plants were identical to the wild-type.

Although full-length transcripts of *At4g21960* and *At4g22250* were not detected in the *inv*/*inv* plants (Figure I-1e), partial transcripts from the fused fragments of *At4g21960* and *At4g22250* were detected in the *inv*/*inv* plants (Figure I-2b). In the case of *At4g22250,* the partial transcripts were rather increased in the plants with the inversion. Taken together, these results from expression analysis suggest that the inversion has an influence on the expression of a wide range of genes.

In the *acl1-3* mutants, the expression patterns of the genes related to the inversion, including *At4g21960* and *At4g22250,* were similar to those of the Col +/+ plants (data not shown).

The *acl1-1* phenotype was enhanced by the inversion

While there was no apparent difference between Col *inv/inv* plants and Col +/+ plants (Figure I-3a, Table I-2) and between *acl1-3 inv/inv* and *acl1-3* +/+ plants (Figure I-5), the comparison of the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants made me realize the difference between these two genotypes. Both the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants exhibited the *acl1-1* phenotype with short inflorescence stems and small curly leaves. However, the rosettes of the *acl1-1 inv/inv* plants appeared slightly smaller than those of the *acl1-1* +/+ plants (Figure I-3a). The height of the *acl1-1* +/+ plants was significantly different to that of the *acl1-1 inv/inv* plants, indicating that the growth defects of the *acl1-1* mutants are enhanced by the inversion (Table I-2).

The reduction of the cell length was significant in *acl1-1* background in all types of tissues examined; cells in the epidermis, the outermost layer of cortex, and the pith (Table I-3, Figure I-4a). The reduction in the size of the epidermal cells was most severe, and the cells in pith also were significantly affected by the *acl1-1* mutation. The *acl1-1 inv/inv* plants exhibited more severe reduction in the cell length than the *acl1-1* +/+ plants. Unlike cells in epidermis and pith, the length of cortex cells was less affected. In addition to the severe reduction in length, the differentiation of cells appeared to be inhibited in the inflorescence stems of the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants.

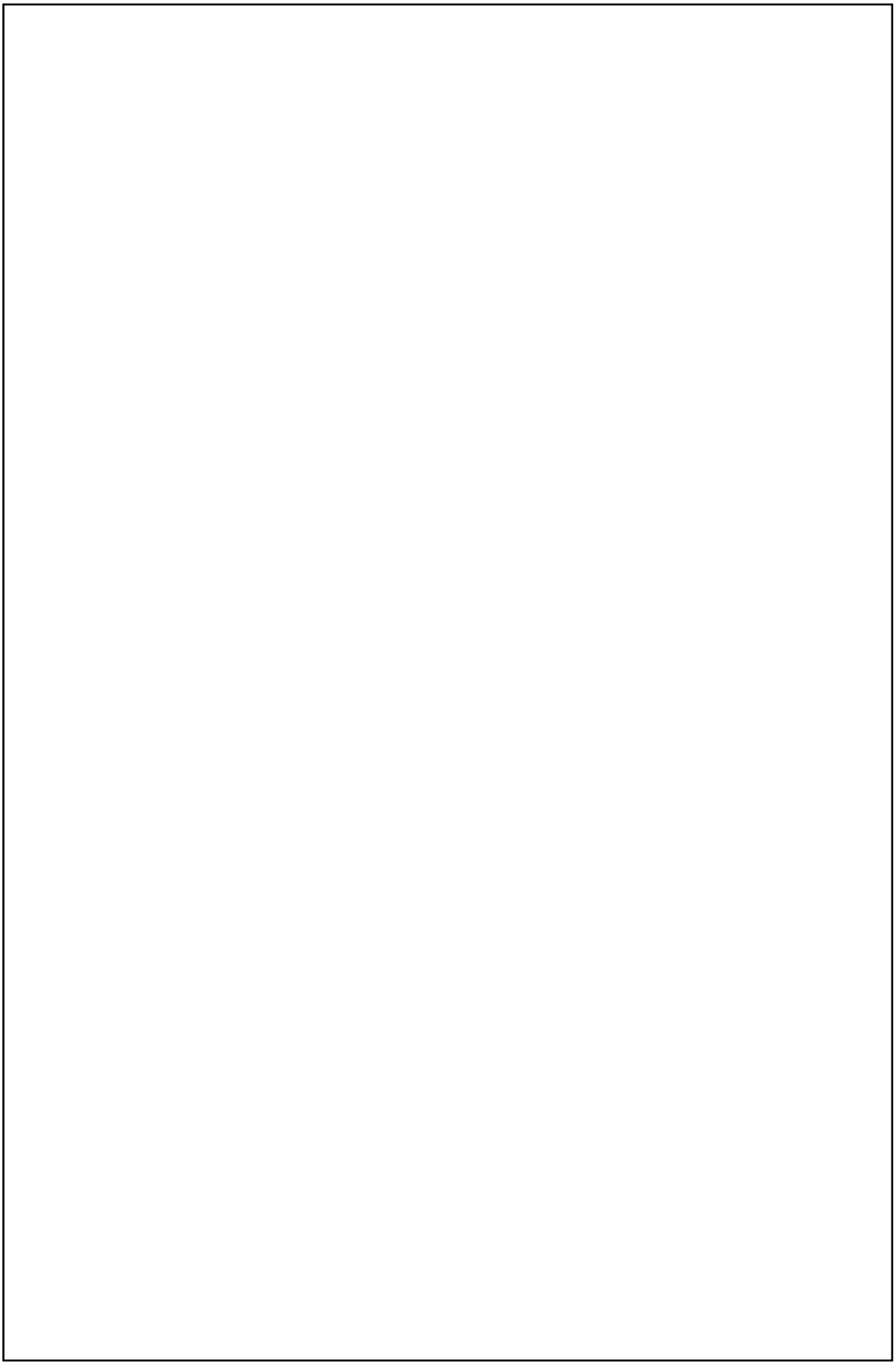
Both the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants were able to restore

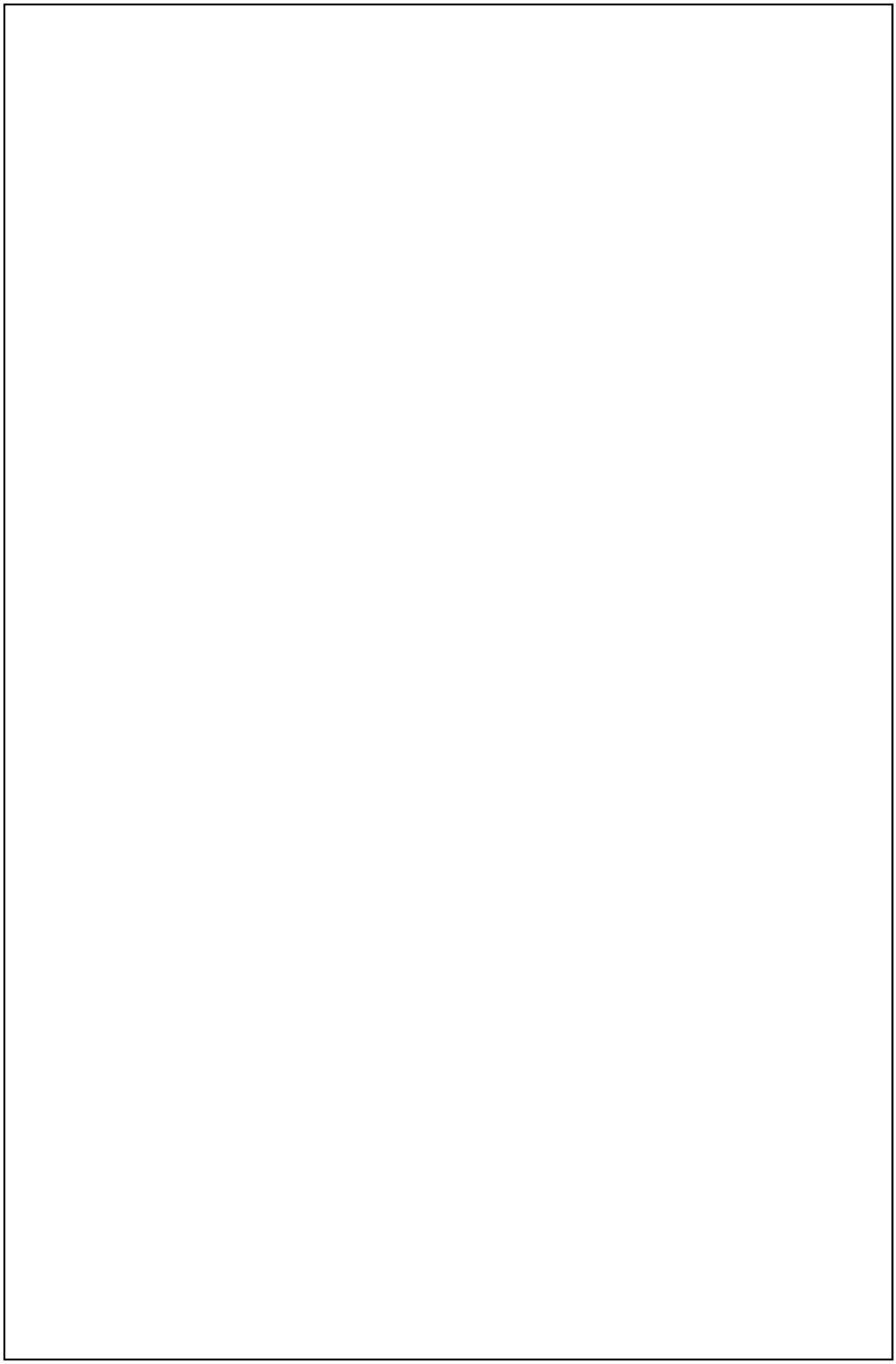
their plant morphology when grown at 28˚C as previously reported (Tsukaya *et al*., 1993). Plants tend to become more slender at higher temperatures, as the result of the general effects of higher temperature. These general effects of higher temperature were observed in the Col *inv/inv* plants as well as in the Col +/+ plants throughout the experiments. Correspondingly, the cells became longer at higher temperature (Figures I-4b,c). The restoration of the *acl1-1* phenotype to wild-type was not complete at the intermediated temperature 24˚C (Figure I-3b). However, the difference between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants became more obvious at 24˚C. The inflorescence stems of *acl1-1* +/+ plants elongated to approximately 5 cm in length (average ± standard deviation, 5.2 ± 3.4 cm, n = 12). On the other hand, the inflorescence stem of *acl1-1 inv/inv* plants was as short as that grown at 22˚C. At 24˚C the length of cells, including those in the cortex, became significantly different between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants (Table I-3). While neither the *acl1-1* +/+ plants nor the *acl1-1 inv/inv* plants fully restored the *acl1-1* phenotype at 24˚C, the inflorescence stems of both the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants elongated to a similar length to those of Col plants at 26˚C and showed complete restoration to wild type (Table I-4, Figures I-4b,c). There was no difference between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants at temperatures exceeding 26˚C ($t = 0.014$ for plant height at 26˚C).

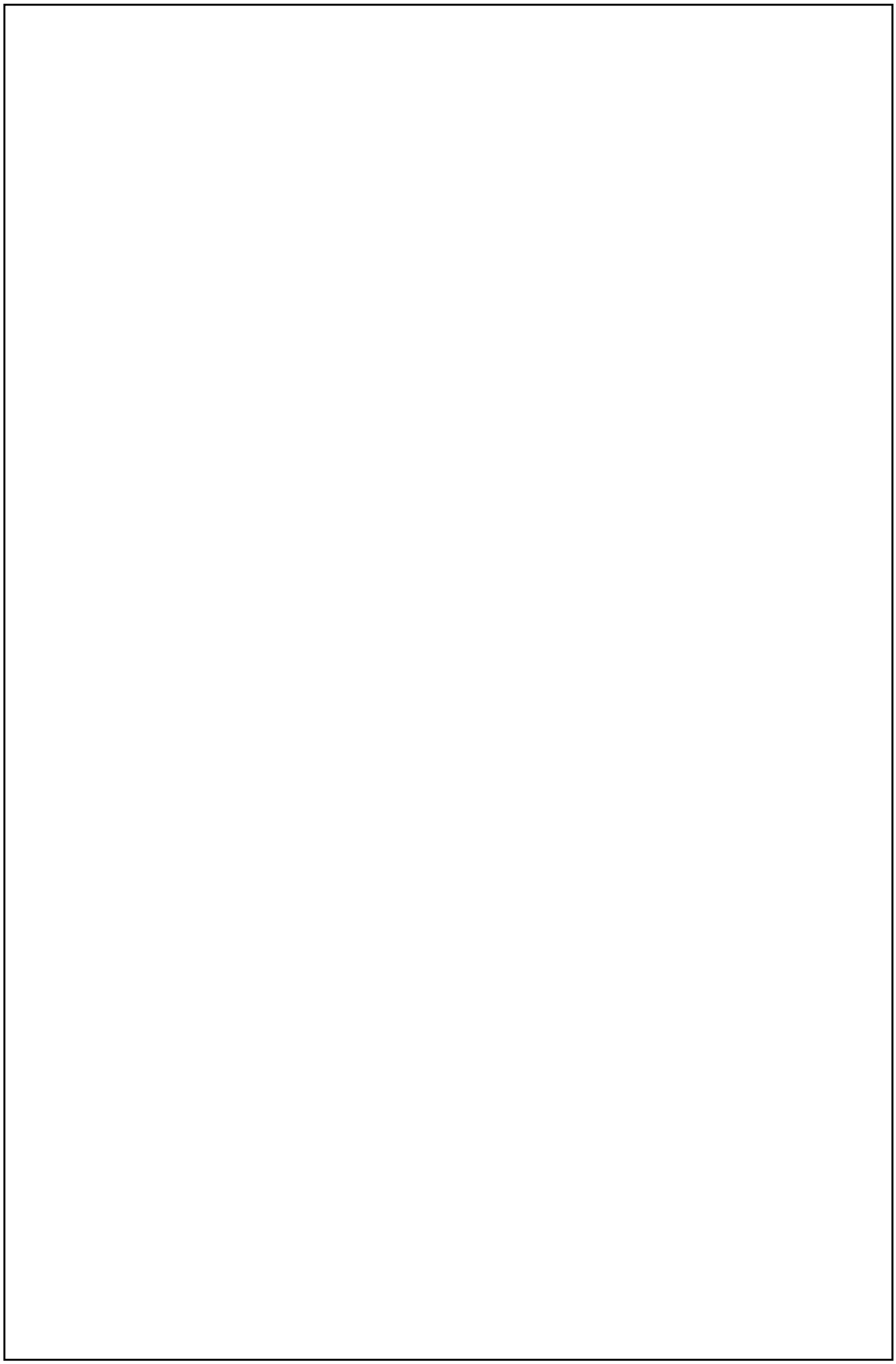
Part 2

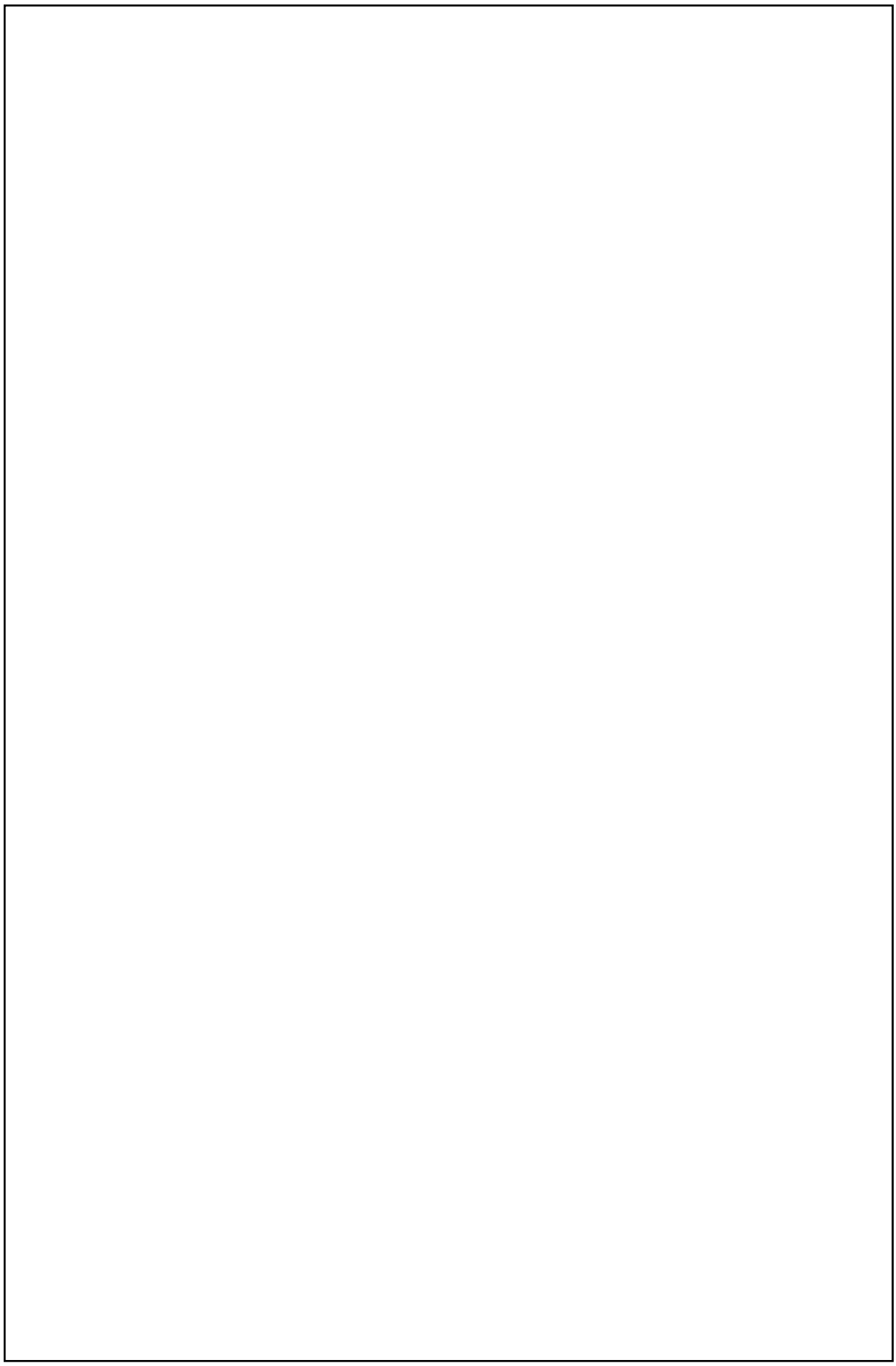
Stunted growth of *acaulis1* (*acl1*) mutants is dependent on constitutive activation of defense response pathways induced by *SUPPRESSOR OF NPR1, CONSTITUTIVE1* (*SNC1*)

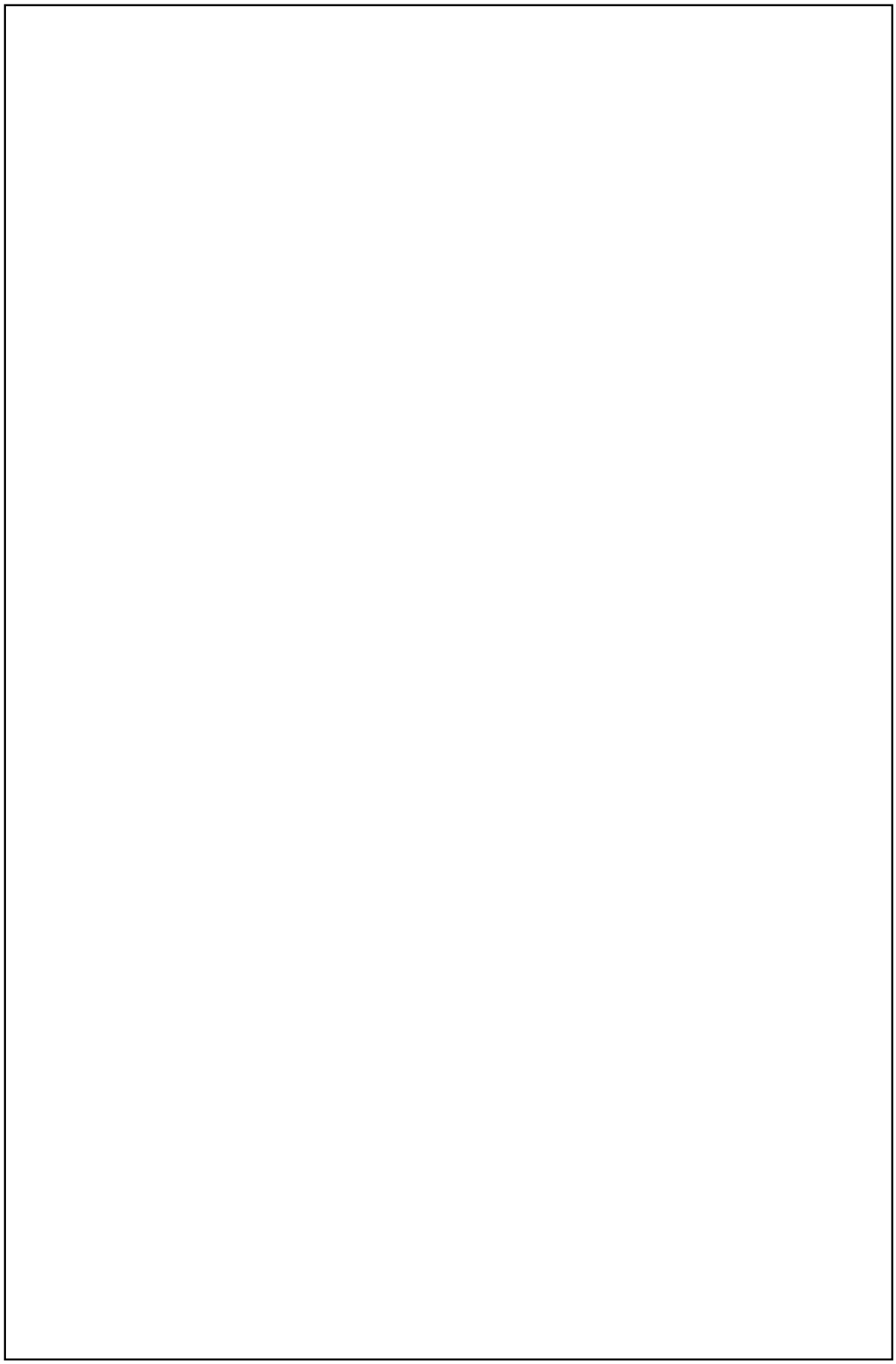
本パートについては最終項を除いて、5年以内に雑誌等で刊行予定のため非公開。

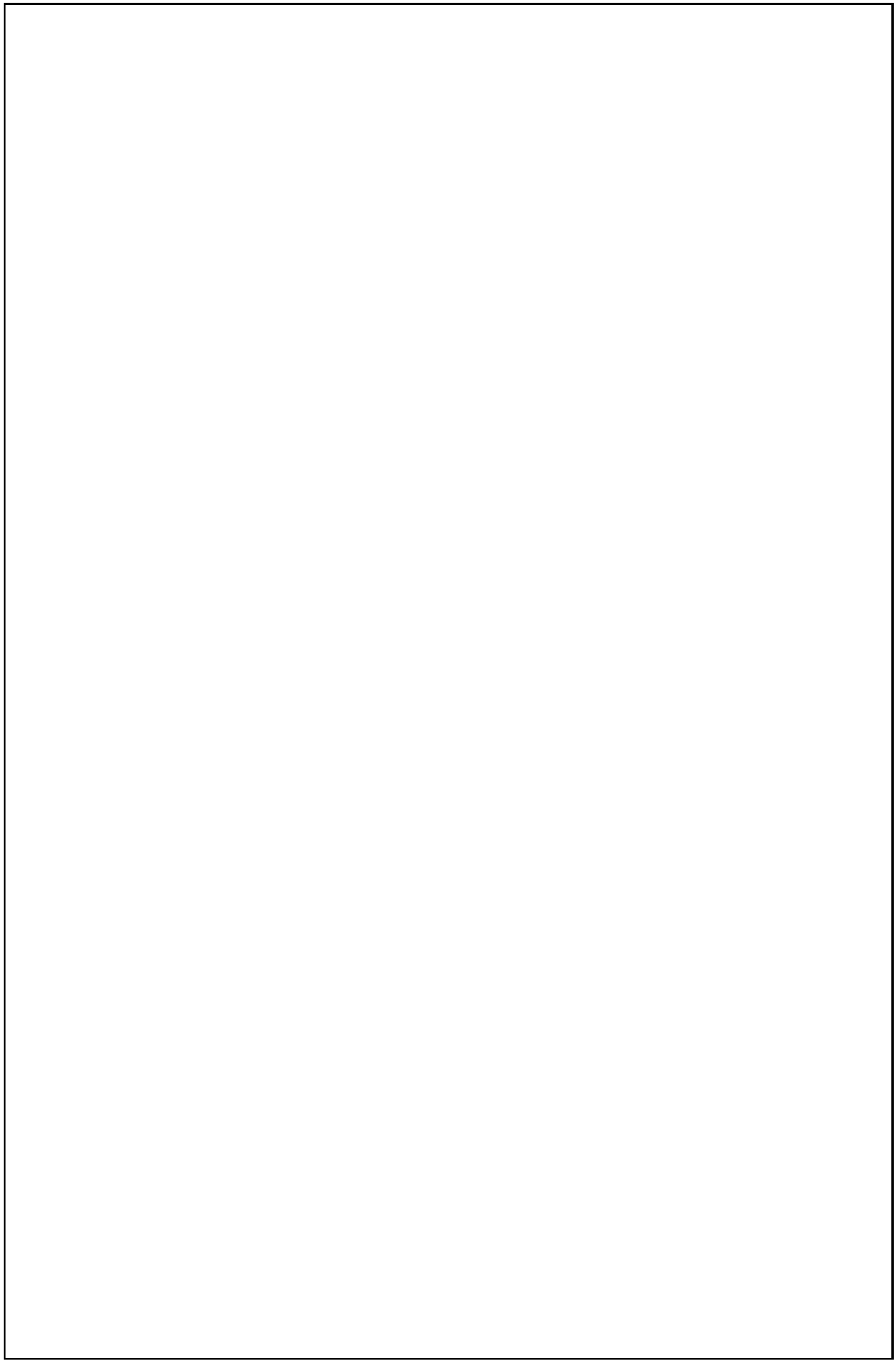


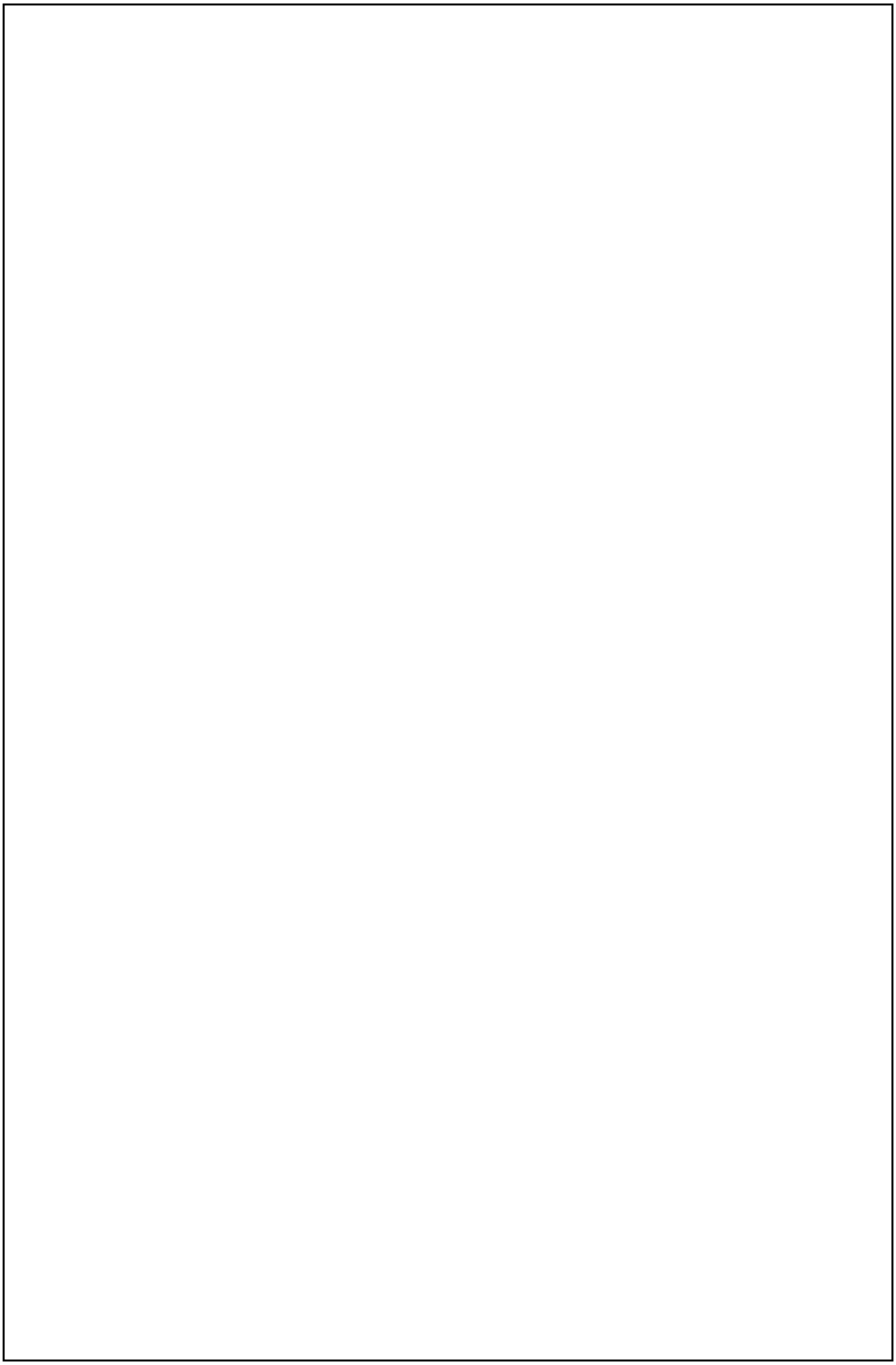


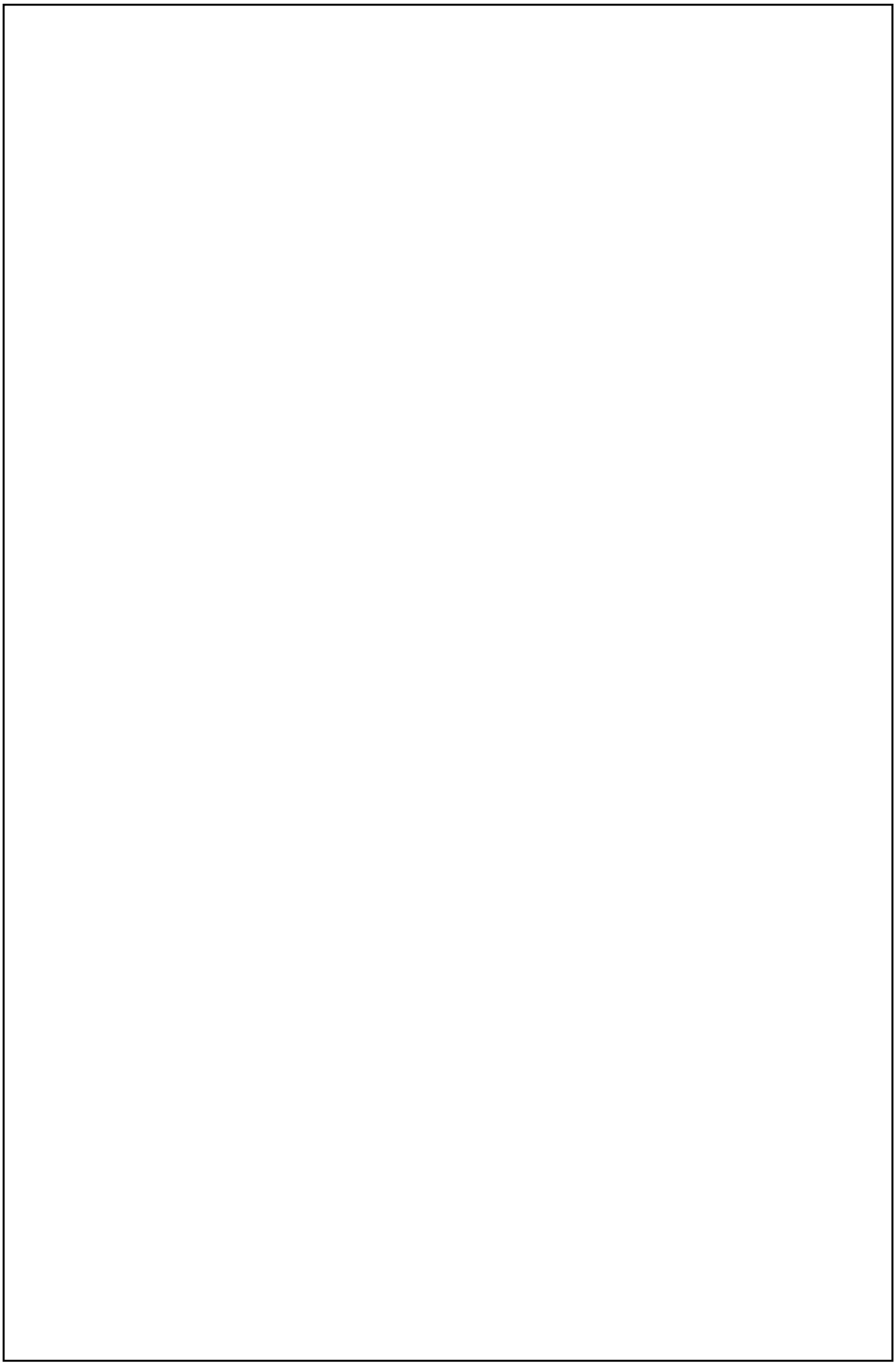












Expression of defense related genes are increased in *acl2-1,* but the *acl2-1* phenotype was not fully suppressed by high temperature and *eds1-2* mutation

acl2-1 is a semi-dominant mutation that causes reduction of stem length and a slight abnormality in leaf morphology (Tsukaya *et al*., 1993). *ACL2* appeared to regulate stem and petiole length via control of cell length for the most part (Tsukaya *et al*., 1995; 2002). *ACL2* was mapped to an interval of <2 cM on chromosome 1. However, the precise effect of *acl2-1* mutation on the plant growth in a molecular level is not yet been confirmed (Tsukaya *et al*., 1995). I observed that the growth defect in the *acl2-1* mutant is alleviated at higher temperatures (Figure I-14a). Although a complete restoration was not observed even at 28˚C, temperature-dependent growth potential of the *acl2-1* mutant suggests a possibility that the *acl2-1* phenotype is also related to the constitutive activation of defense responses similar to the *acl1* mutants. By expression analysis, *PR1* and *PR2* were found to be highly expressed in the *acl2-1* mutant, while the key regulatory genes essential for *R* gene-mediated defense response, *EDS1*, *PAD4* and *NDR1,* were only moderately increased (Figure I-14b). Different from the *acl1* mutants, the *PR1* and *PR2* expression were not obviously altered by the increases in temperature, suggesting that activation of defense responses in the *acl2-1* mutant is more tolerant to higher temperatures than those in the *acl1* mutants.

The growth defect was most restored in *acl2-1 eds1-2* double mutant, though the restoration was not complete, among the double mutants generated between *acl2-1*

and mutants of key regulatory genes in defense responses (Figure I-14c). Plant growth of *acl2-1 pad4-1* and *acl2-1 ndr1-1* double mutants was also restored to some degree, suggesting that the *acl2-1* phenotype is partially dependent on *EDS1* and, less on *PAD4* and *NDR1*. *acl2-1 sid2-2* double mutant slightly restored the mutant phenotype, implying a more or less influence of SA on the *acl2-1* growth. However, *npr1-1* mutation had no effect on the *acl2-1* phenotype.

I further examined the *acl2-1* mutant growth on different nitrogen conditions. However, different from the *acl1* mutants, the *acl2-1* mutants grown on the higher concentration of ammonium were not so much altered from those grown on low concentration of ammonium, and nitrogen condition had no striking effects on the *acl2-1* growth (Figure I-14d,e).

Discussions

In the part 1, I isolated the "genuine" *acl1-1* plants without the inversion. By comparing these plants to the original *acl1-1* plants with the inversion, the inversion was likely to be function as an enhancer of the *acl1-1* phenotype.

The inversion enhanced the *acl1-1* phenotype and altered expression patterns of various genes

In the previous study, there was a description that the length of the inflorescence stems of *acl1-1* plants varied among plants, even though they were grown

side by side under the same conditions at 22˚C (Tsukaya *et al.,* 1993). It is possible that this unknown factor that influences the growth of the original *acl1-1* mutants is the inversion identified in this study. I observed that the height of plants, the size of rosettes, and the length of cells were remarkably reduced in the *acl1-1 inv/inv* plants compared with the *acl1-1* +/+ plants at 22˚C (Tables I-2 and I-3, Figures I-3 and I-4). Moreover, the inversion functioned more effectively as an enhancer of the *acl1-1* phenotype when plants were grown at 24˚C, inhibiting partial restoration of the *acl1-1* phenotype in the *acl1-1 inv/inv* plants (Figures I-3b and I-4b,c). When the *acl1-1* phenotype was fully restored to wild-type at 26˚C or 28˚C, the difference between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants disappeared. Considering that the inversion itself was not sufficient to significantly alter plant morphology in the Col-0 and *acl1-3* backgrounds (Table I-2, Figures I-4 and I-5), it seems that the inversion enhances the defects in the plant growth only when severe growth defects are already present, such as in *acl1-1* plants.

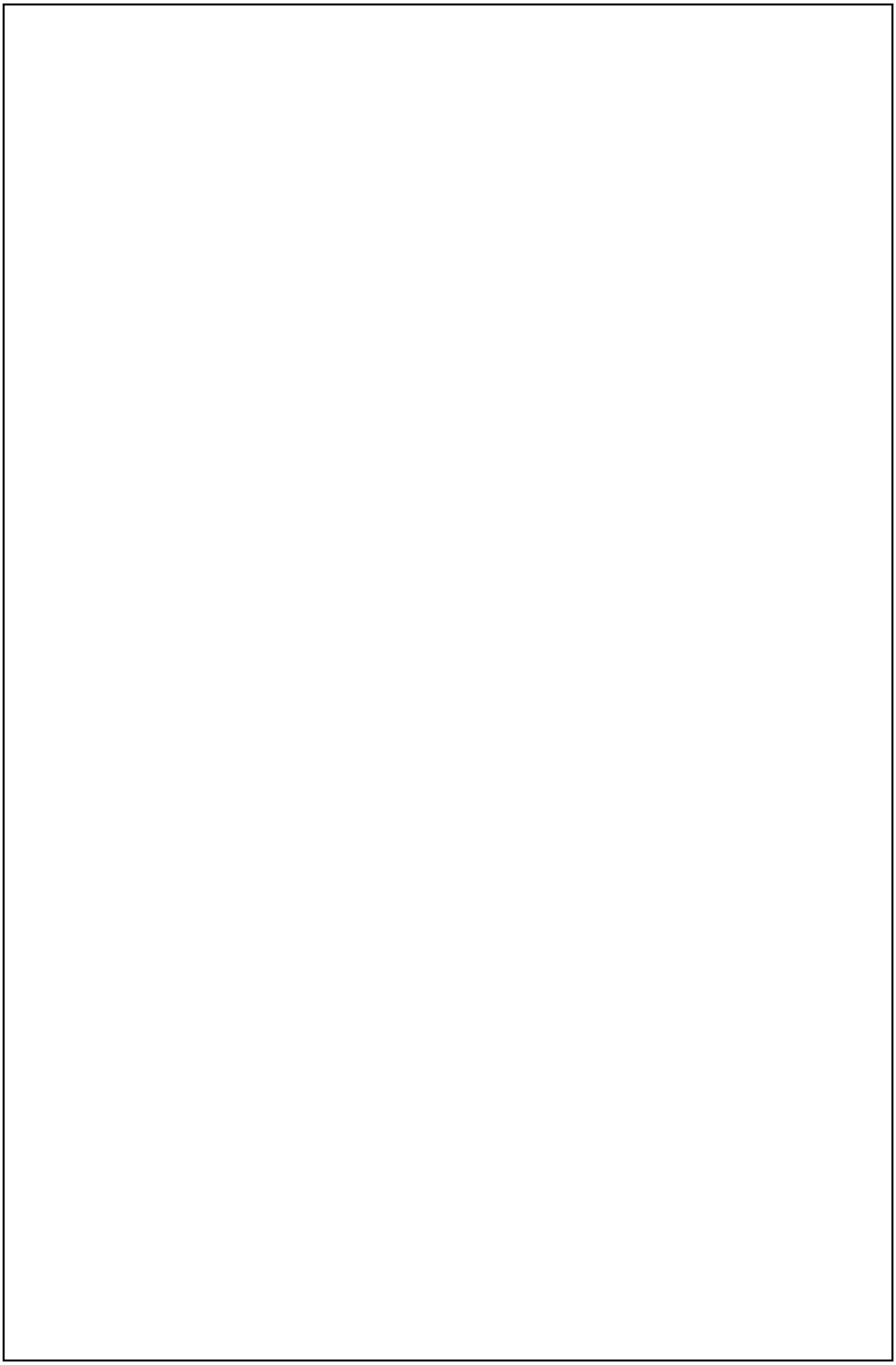
I found that, in addition to loss of full-length transcripts of *At4g21960* and *At4g22250,* the expression patterns of some other genes within and adjacent to the inversion and were altered by the inversion (Figure I-2). Although it is not yet confirmed, it is possible that the genes distant from the inversion are also affected by the inversion and that organ-specific and/or age-specific expression is altered. I propose that multiple genes are complicatedly involved in the enhancement of the *acl1-1* phenotype.

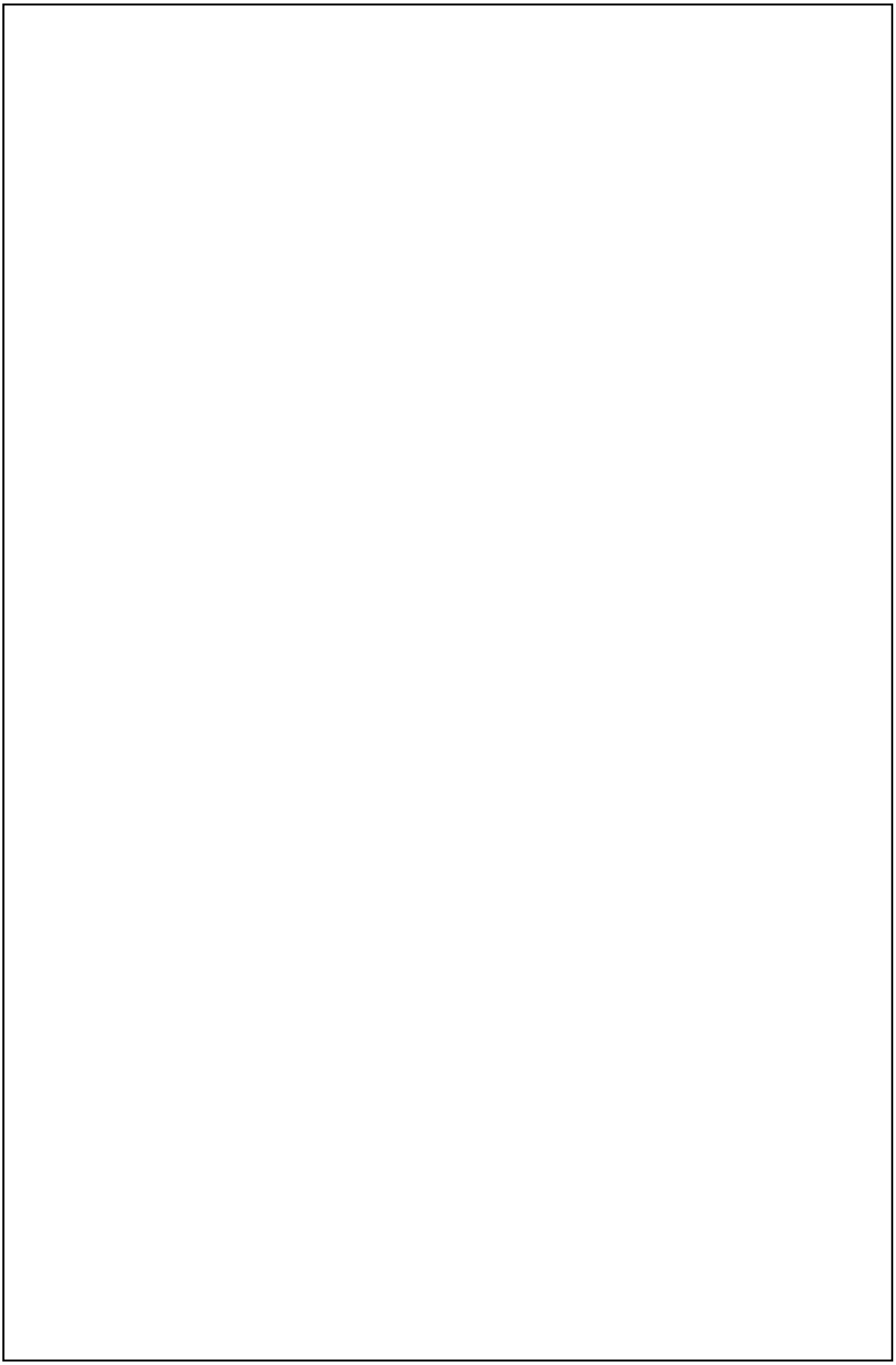
The original *acl1-1* mutant was obtained by X-ray irradiation. In most higher

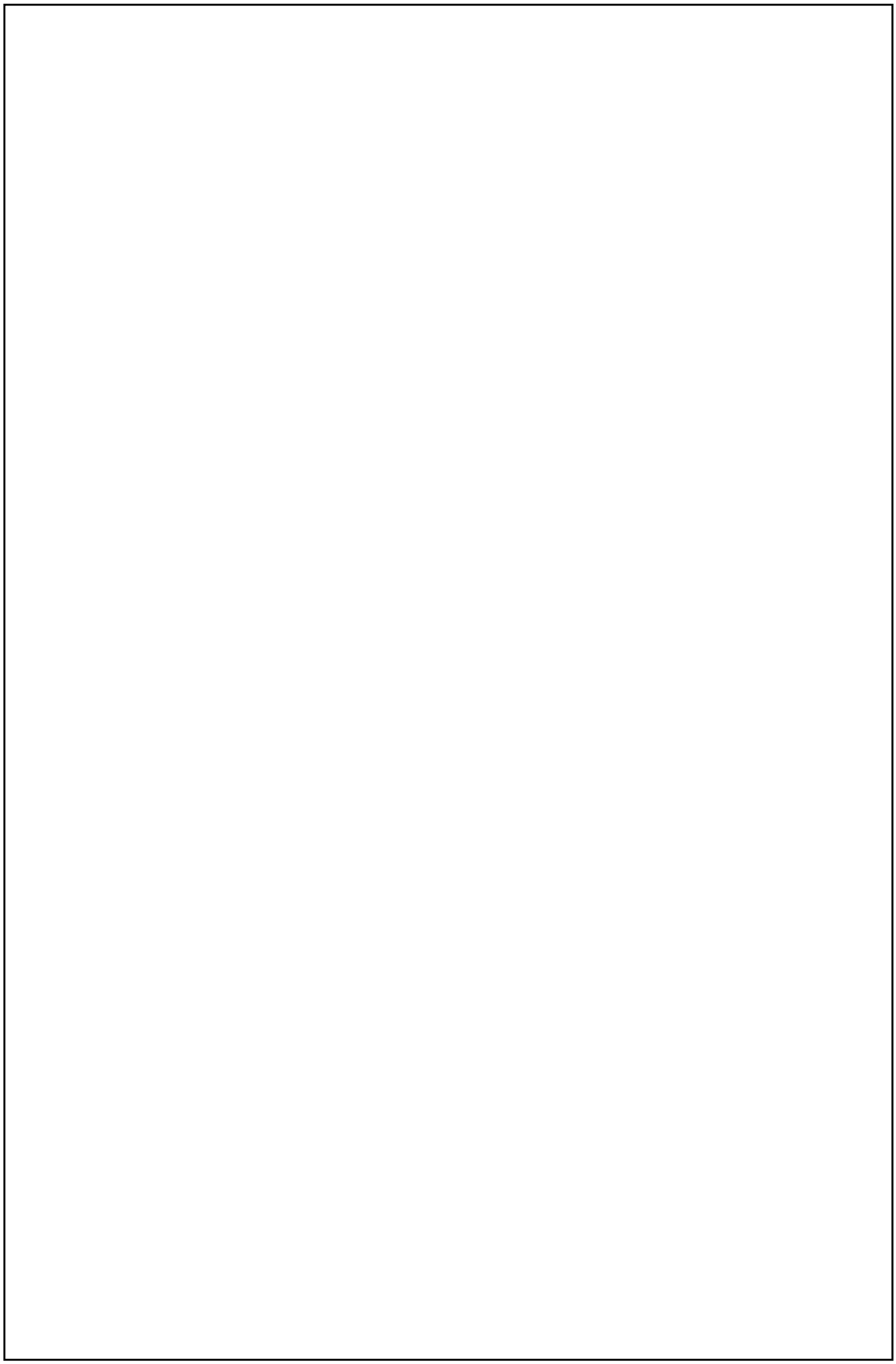
plants, including *Arabidopsis*, DNA double-strand breaks caused by ionizing irradiation (fast neutron, X-ray, and γ-ray) are predominantly repaired by non-homologous end-joining rather than by simple ligation or accurate homologous recombination. In non-homologous end-joining, any end can fuse with any end. Thus, the repair of double-strand breaks in plants is suggested to be error-prone (Gorbunova and Levy, 1999). Sometimes, the repair of double-strands breaks has very complex DNA rearrangements, combining deletions, insertions, inversions and duplications of the original sequence (Shirley *et al*., 1992), and that was what was confirmed at the border regions of the inversion isolated from the original *acl1-1* (Figure I-1d).

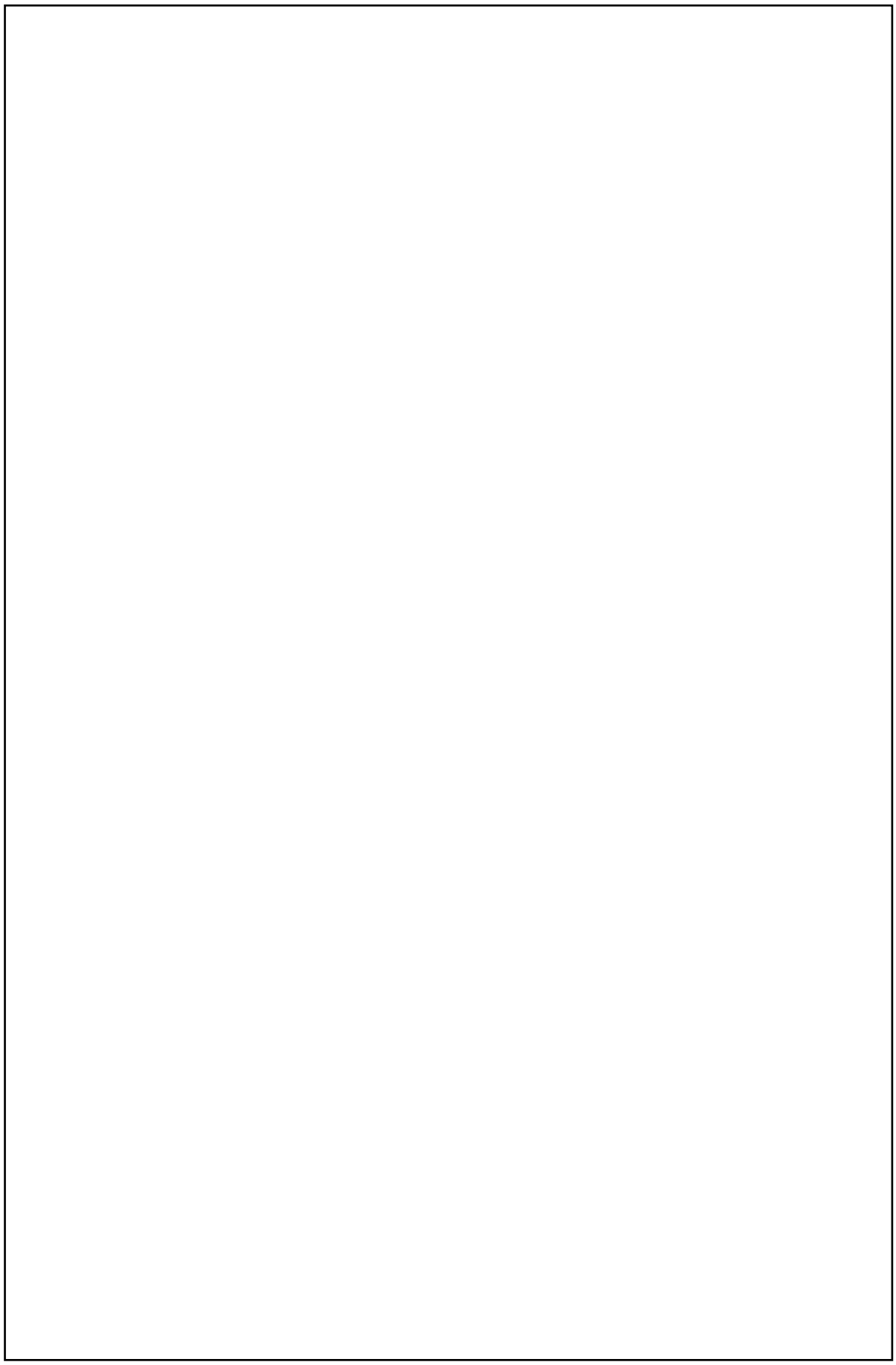
In the part 2, further studies on the *acl1* mutants were carried out using the *acl1-1* +/+ line isolated in the part 1.

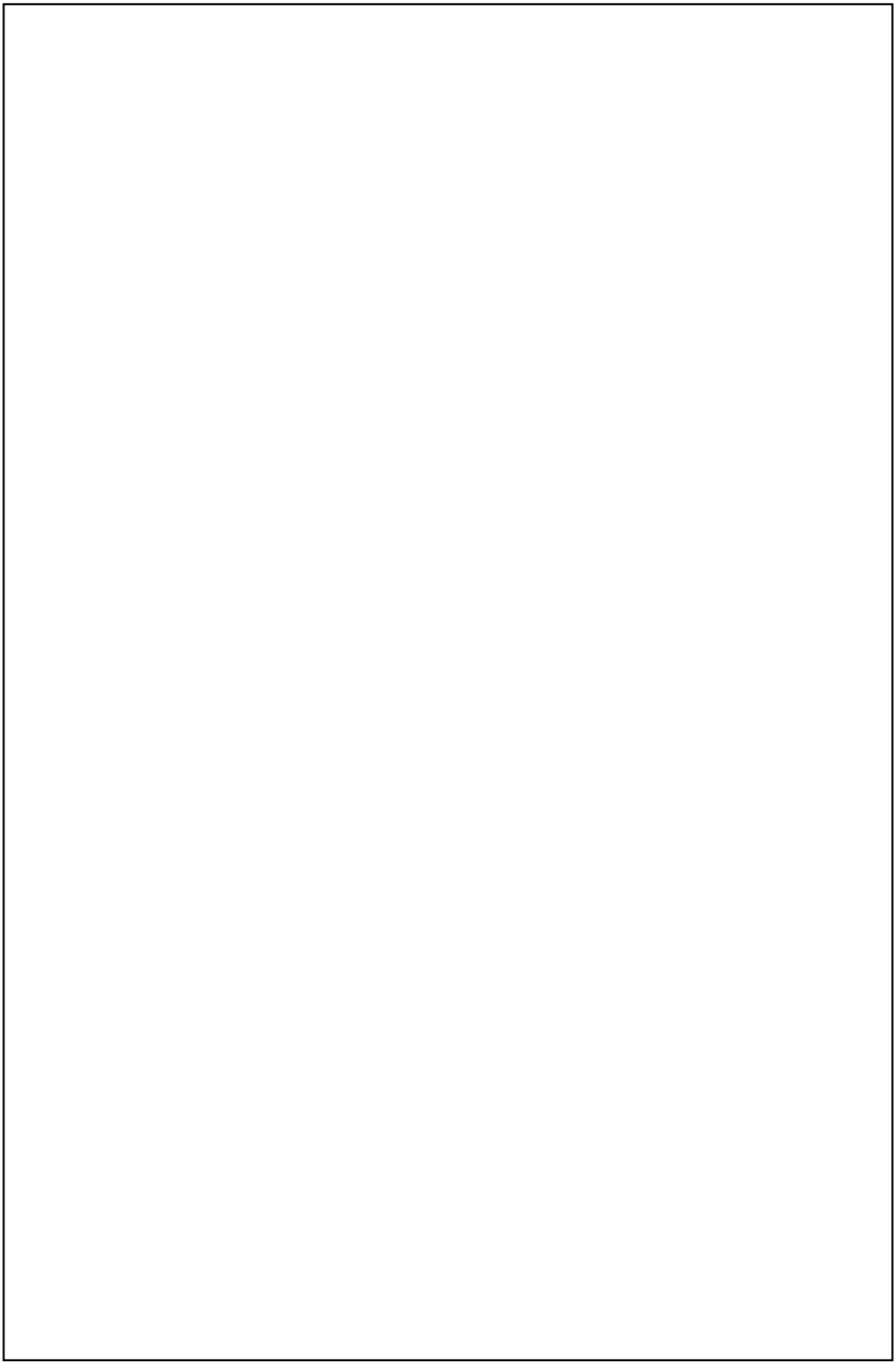
5年以内に雑誌等で刊行予定のため非公開。

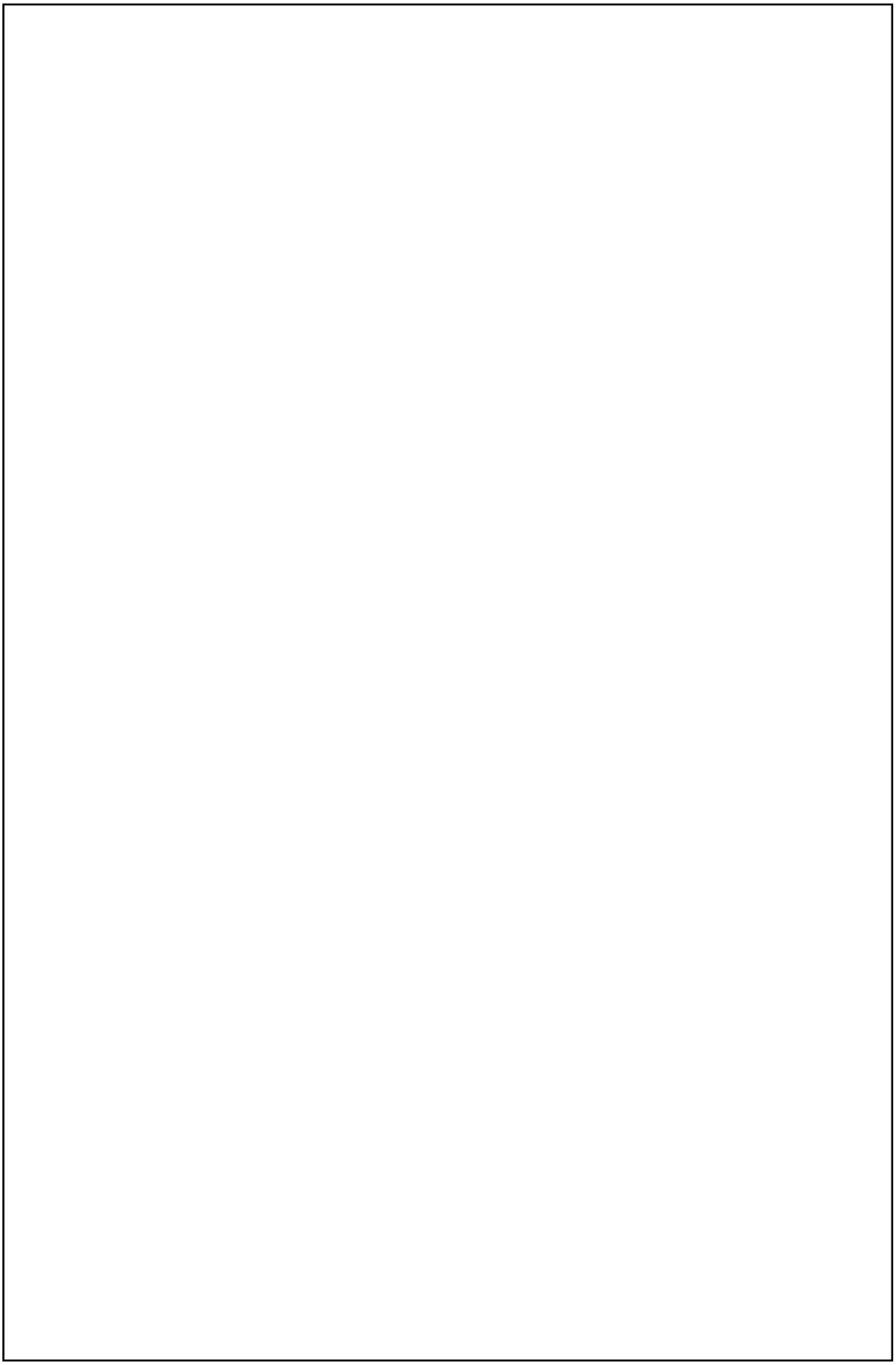


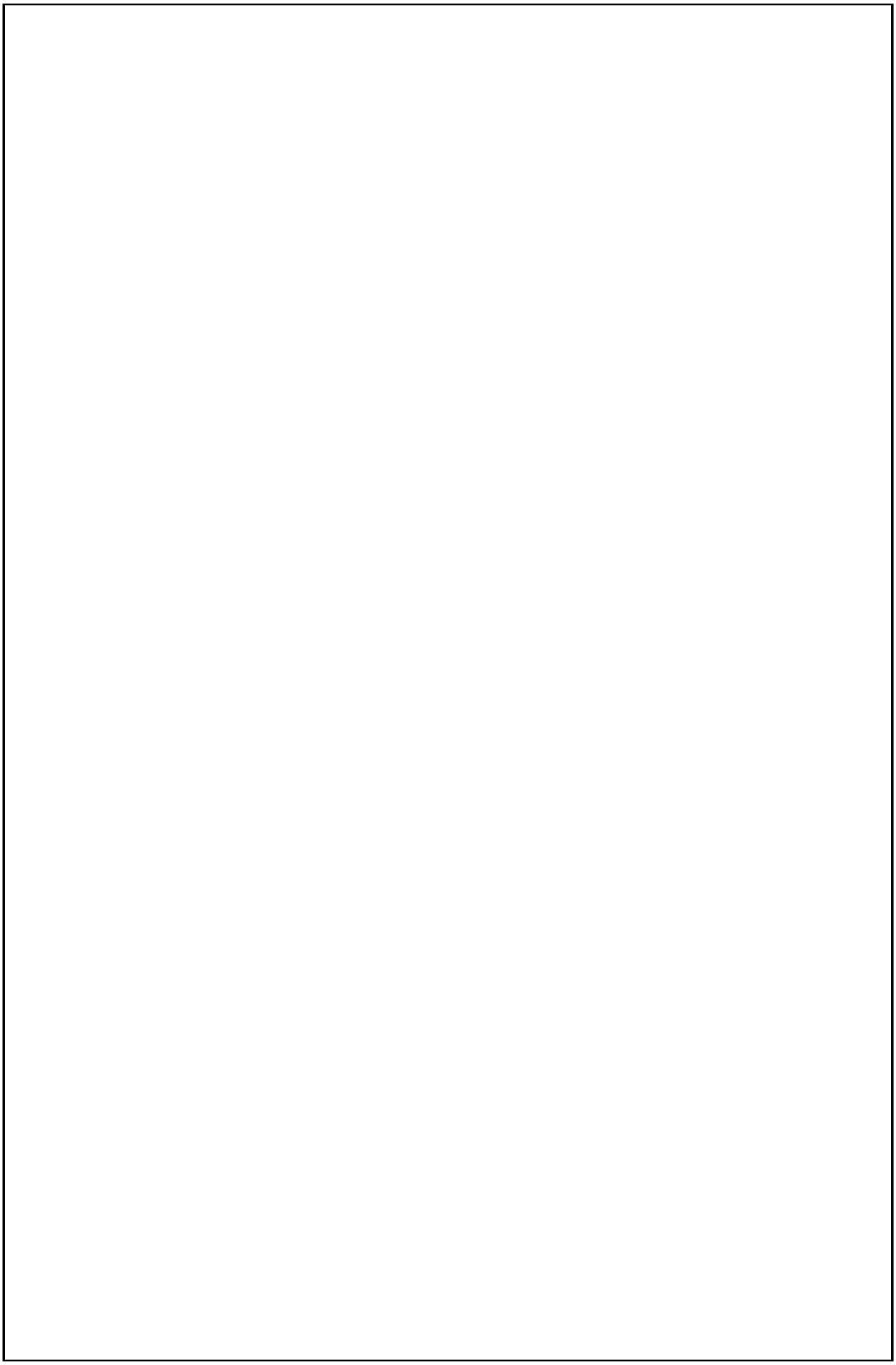


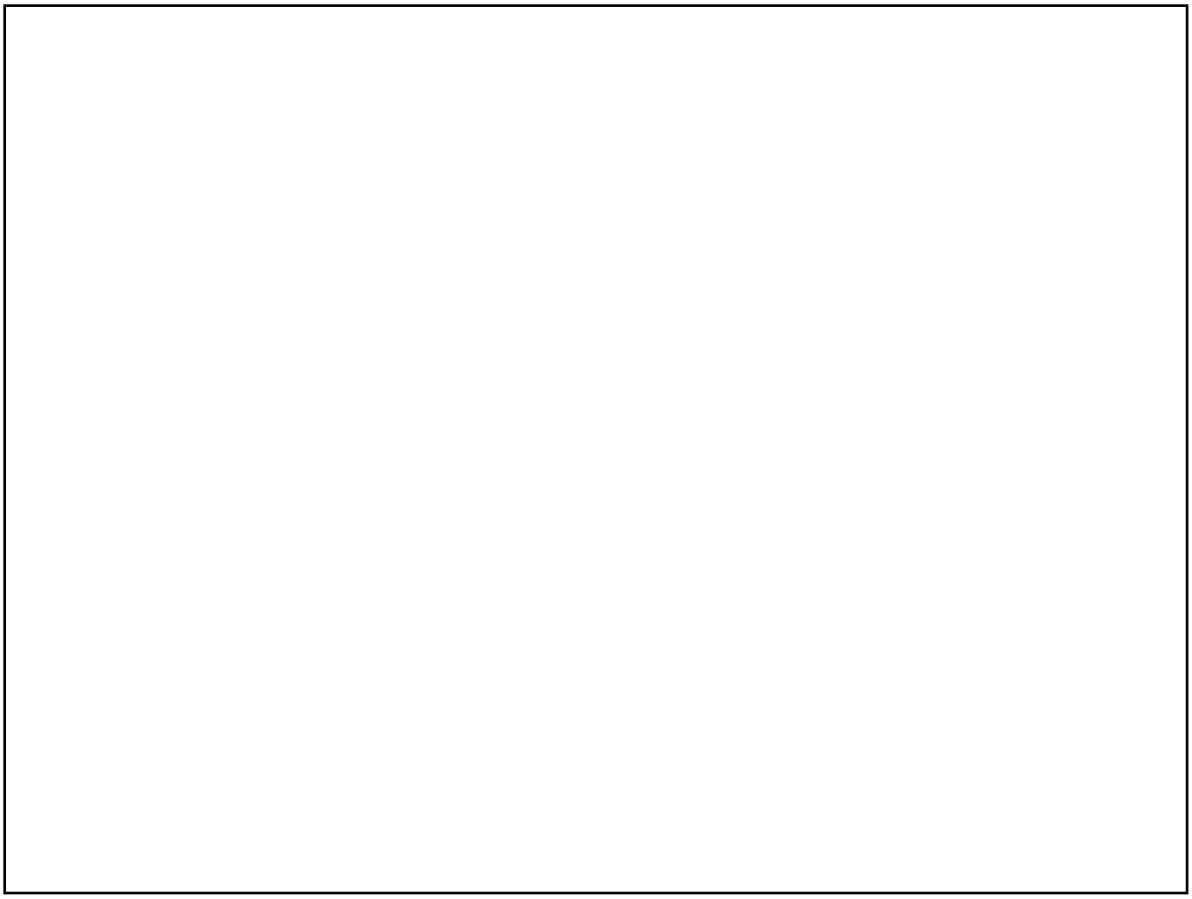












The *acl2* mutation causes constitutive activation of defense response pathways

As shown in Figure I-14b, the *acl2-1* mutant showed increased expression of defense related genes, suggesting the constitutive activation of defense responses. However, different from the *acl1* mutants, the trigger of defense responses in *acl12-1* seems to be more tolerant to higher temperatures, since 28˚C was not sufficient to fully repress the elevated expression of defense related genes in *acl2-1* (Figure I-14a,b). Also, the *acl2-1* growth phenotype was not completely restored neither by *EDS1* nor *NDR1* (Figure I-14c). Considering an early termination of inflorescence stem growth associated with defense responses that is independent of *EDS1* and *NDR1*, the *acl2-1* mutation might have something in common with *uni-1d*, which caused by a

gain-of-function mutation of CC-TIR-LRR type or *R* gene (Igari *et al*., 2008). *uni-1d* phenotype requires ER receptor kinase family members (Uchida *et al*., 2011a) and, in fact, inflorescence elongation of the *acl2-1* mutant alleviated in L*er* background (Prof. Atsushi Kato, personal communication). The uni-1D/UNI protein interacts with regulatory particle triple-ATPase (RPT) subunit 2a of the 19S regulatory particle in the 26S proteasome, which is turned out to be responsible for inducing both defects in morphology and defense responses (Chung and Tasaka, 2011), although I am not sure for the involvement of RPT2a in the *acl2-1* mutant phenotype at this time. Further investigations on the genetic factors involved in the *acl2-1* phenotype and on the *acl2-1* mutation itself are needed to understand the mechanisms regulating the plant growth and defense responses in the *acl2-1* mutant.

Materials and Methods

Plant lines

Arabidopsis thaliana accession Columbia (Col-0) was used as the wild type. *acl1-1* (Tsukaya *et al*., 1993; Kamata and Komeda, 2008), *acl1-3, acl2-1* (Tsukaya *et al*., 1993), *pad4-1* (Glazebrook *et al*., 1996), *ndr1-1* (Century *et al*., 1995), *npr1-1* (Cao *et al*., 1994), *rar1-21* (Tornero *et al*., 2002), *sid2-2* (Wildermuth *et al*., 2001), *snc1-11* (SALK_047058) and *rpp4* (SALK_017521) (Yang and Hua, 2004) mutants were previously described. The *eds1-2* mutation introgressed into Col-0 background was used as the *eds1-2* mutant in this study (Parker *et al*., 1996). *2080* (*acl1-4*) was isolated from

the pool of T-DNA insertion mutants, by Goro Horiguch, and kindly gifted. 2080 was backcrossed to Col-0 for more than 5 times. Backcrosses indicated that the *2080* phenotype is caused by a recessive mutation at a single genetic locus, which does not link with the T-DNA insertion. *2080* was found to be allelic with *acl1-1* and *acl1-3* by the phenotype of F1 plants between them. Transgenic line SGT754-5-3 having a T-DNA insertion in the 3rd intron of *At4g21960* (La background), and SALK_018861 and SALK_044071 lines having T-DNA inserted in the promoter region and 3'UTR of *At4g22250* respectively (Both are Col background) were isolated from Salk T-DNA lines (Alonso *et al*., 2003).

To obtain double mutants, *acl1* mutants were crossed with each defense mutant. F2 plants expressing the *acl1* phenotype and heterozygous to the defense mutation were selected and then self-fertilized. PCR primers used for genotyping defense genes are listed in Table I-9.

Plant growth

Seeds were sown on water-moistened rockwool, which placed on vermiculite in pots, and placed in darkness at 4˚C for 3 days before they were transferred to growth chambers. Unless noted, the growth chambers were set at 22˚C under long-day conditions (16 hours light/ 8 hours darkness) and plants were watered with the modified MGRL medium (Kamata and Komeda, 2008), which contains 10 mM nitrate and 0.5 mM NH_4Cl as nitrogen source. For plants grown on plates, surface-sterilized seeds were sown on the media containing 2 % (w/v) sucrose and 0.8% (w/v) bacto agar (BD).

Growth temperature was altered to 24˚C, 26˚C or 28˚C and MS (Wako), 1/2X MS, nitrogen-free medium was used if necessary. The nitrogen-free medium contained 5 mM $CaCl_2$ instead of 5 mM $Ca(NO_3)_2$ and NH_4Cl was excluded from the MGRL medium. Media were additionally supplemented with nutrients as described in the text.

Map-based cloning of *acl1*

acl1 mutants were crossed to L*er* and F2 to F4 progenies exhibiting the *acl1* phenotype were used for recombination analysis using cleaved-amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers based on the information from the Arabidopsis Information Resource (TAIR, www.arabidopsis.org). Additional derived CAPS (dCAPS) markers at polymorphisms between Col and L*er* accessions, designed based on information available from TAIR (Table I-7). The 159 Kb region including *RPP5* gene cluster was amplified separately as short overlapping fragments by PCR (Ex taq, Takara Bio) and sequenced with BigDye® Terminator v.1.1 Cycle Sequencing kit (Applied Biosystem).

Examination of the inversion

Genomic DNA was extracted from rosette leaves as described by Edwards *et al*. (1991). Primers used for the examination of the existence of genomic region from *At4g22290* to *At4g21690* are designed more than 50 bp outside from the gene regions annotated by TAIR. Expected PCR fragments were less than 7 kb and amplified with Ex taq (Takara Bio). PCR fragments were then treated with several restriction enzymes and cleaved

into shorter fragments to identify the changes in the length of fragment more precisely by agarose gel electrophoresis. In the case of genes longer than 7 kb, genes were separated in two parts to ensure the amplification by PCR. For the amplification of *At4g21960*, NK215; 5'-GAGAT CAGTA AAATA GATCG-3' and NK216; 5'-TTTAA GGAGC GTGCA TTGC-3' were used as primers. For *At4g22250*, NK232; 5'-TATAA TGTCA TCATC ACTGC-3' and NK240; 5'-TCGAG TATCT CAATG ATCGG-3' and for *At4g21920*, NK 248; 5'-AAACA TCAAA CTTCA CGGAG-3' and NK249; 5'-AATAC GTAGT TTTGA CCTGG-3' were used. PCR fragments of approximately 2.2 Kbp, 0.8 Kbp and 3.3 Kbp in length were obtained by PCR respectively. NK255 5'-AGATC ACATT GAATC TGCAG-3' and NK256 5'-TAAGT CAGTG TGGAA CTAAG-3' were designed at non-gene-coding region between *At4g21960* and *At4g21970* to obtain 1.7 Kbp PCR products used just for the positive control of PCR performed in Figure I-1. For detecting the conjugated fragments of *At4g21960* and *At4g22250,* NK215 and NK240 were used to obtain approximately 1.3 Kbp PCR fragment fragment and NK216 and NK232 were used to obtain approximately 1.6 Kbp PCR fragment only from the inversed DNA. Conjugated fragments were sequenced with BigDye® Terminator v.1.1 Cycle Sequencing kit (Applied Biosystem).

Expression analysis for genes related to the inversion mutation.

Plants were grown for 10 days on the agar plate at 22˚C under long-day conditions. RNA was extracted with RNeasy® plant mini kit (QIAGEN). Extracted RNA was treated with cloned DNaseI (Takara Bio). Reverse-transcriptase reaction was carried out

with Random 9 mers to synthesize cDNA from every type of transcripts. Both RT (reverse transcriptase) reaction and PCR (polymerase chain reaction) were preformed with RNA PCR kit (AMV) Ver.3.0 (Takara Bio). Gene specific primers used for PCR were NK244; 5'-ACTGC GCGGT GGAGT CATG-3', NK856; 5'-GTAGC ATGTG AGGGA CGTGG-3', NK398; 5'-AGACG GAGAT TCCAG GTTG-3', NK239; 5'-TTAGA GTTTC CGTTA CCGAG-3', NK349; 5'-CTAGG AGGGC GACGA GGC-3', NK262; 5'-TTTAT GACAC GTGCA GGG-3, NK350; 5'-TCGTG ACCGC TCATC TGTC'-3', NK263; 5'-GTGCT CTAGA GAATT GTGGC-3'. *EF1a* (*At5g60390,* as a control) were amplified with NK24; 5'-ACTTG CAGCT ATGGG TAAAG-3' and NK25; 5'-CGAAA GTCTC ATCAT TTGGC-3'. For semi-quantitative RT-PCR of the genes located near and within the inversion, primers listed on the Table I-10 were used.

Semi-quantitative RT-PCR for genes related to defense responses

Total RNA from the shoot of 10 day-old seedlings grown on sterile agar plates was extracted using RNeasy plant mini kit (Qiagen). The first-strand cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction volume using the PrimeScript RT-PCR kit (Takara Bio) with Oligo-dT primer. Sequences of gene-specific PCR primers are provided in Table I-11. For Semi-quantitative RT-PCR analysis, the primers were designed to span introns to avoid amplification from contaminated genomic DNA, except for *At4g16880*. PCR runs consisted of 24-32 cycles, depending on the linear range of PCR amplification for individual genes. PCR cycle included incubations at

94˚C for 30 sec, at 55˚C for 30 sec, and at 72˚C for 90 sec. PCR products were detected by electrophoresis through 1.2% agarose gels, depending on the length of PCR product and stained with ethidium bromide. All RT-PCR procedures were repeated at least three times with similar results.

Observation of cellular structure and measurement of cell length

Inflorescence stems of plants grown for 40 days after germination were fixed overnight in FAA [70% ethanol: formaldehyde: acetic acid = 18: 1: 1 (v/v)], dehydrated in an ethanol series and embedded in Technovit resin as described in manual (Kluzer). 5 μm thick sections were stained with 0.05% (w/v) toluidine blue O dissolved in 1% (w/v) $Na_2BO_4O_7$ $10H_2O$ aquous solution. Sections were photographed (E-330, Olympus) under light microscopy (eclipse 80i, Nikon) and cell length was measured using Photoshop 8.0 (Adobe systems). Three individuals from each strain and each temperature were used for analysis.

Trypan blue staining

Leaves were stained with lactophenol trypan blue solution [10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue dissolved in 10 ml of distilled water] by boiling for approximately 1 min. Leaves were then cleared in 2.5 g/ml chloral hydrate solution and examined under a light microscope. Dead cells are stained blue.

Preparation of the genomic DNA library and next-generation sequencing

1 g of frozen seedlings of Col, *acl1-1* and *acl1-3* were individually ground in liquid nitrogen to a fine powder. Nuclei fraction was enriched using "Semi-pure Preparation of nuclei procedure" of CelLytic PN Isolation/Extraction Kit (Sigma-Aldrich), and genomic DNA was isolated using Plant DNeasy mini kit (Qiagen). 0.5 μg of DNA was sheared using Covaris S2 (Covaris) at 100-bp setting. After being purified using a QIAquick PCR purification kit (Qiagen), the DNA library was prepared using Genomic Adaptor Oligo Mix (Illumina) as the DNA adaptor and NEBNext DNA Sample prep Reagent Set 1 (New England Biolabs) according to manufacturer's manual. Ligation products were size-selected by electrophoresis on 2% (w/v) agarose gel. 200-250 bp DNA fragments were excised from agarose gel and purified using QIAquick gel extraction kit (Qiagen). Then adopter-modified DNA fragments were enriched by PCR with PCR Primers 1.1 and 2.1 (Illumina) and KAPA HiFi HotStart ReadyMix (KAPA). The PCR program was 98˚C for 30 sec, followed by 10 cycles of 98˚C for 10 sec, 65˚C for 30 sec and 70˚C for 30 sec. PCR products were gel-purified using a QIAquick gel extraction kit. Sequencing with illumina-GAIIx and informatics were performed as described in Uchida *et al*. (2011b).

Tables

Table I-1. Segregation of *acl1-1* phenotype and the inversion

Phenotype	Inversion		
	Wild type (+/+)	Hemizygote (*inv*/+)	Homozygote (*inv*/*inv*)
Wild-type	123	203	45
acl1-1	5	22	119

Table I-2. Measurement of plant height at 22˚C

Genotype	Average ± standard deviation (cm)			t-value
acl1-1 +/+*	6.0	± 1.8	(n = 39)	
*acl1-1 inv/inv**	4.3	± 1.2	(n = 31)	t = 4.52†
Col +/+**	179.7	± 17.4	(n = 16)	
Col *inv/inv***	180.0	± 29.1	(n = 7)	t = 0.031††

Plants were grown for 40 days.

*Total plant height including the rosette and the inflorescence stem.

**Length of the inflorescence stem was measured as plant height.

†Difference between *acl1-1* +/+ and *acl1-1 inv/inv* is significant (significance level: $\alpha = 0.05$).

†† Difference between Col +/+ and Col *inv/inv* is not significant (significance level: $\alpha = 0.05$).

Table I-3. Comparison between cell length of +/+ and *inv/inv* plants

		Epidermis (μm)	Cortex (μm)	Pith (μm)	n*
22˚C *acl1-1* +/+		43.0 ± 14.6	28.5 ± 6.38	78.0 ± 25.0	50
22˚C *acl1-1 inv/inv*		21.2 ± 9.25	26.1 ± 6.84	43.6 ± 11.1	20
	t-value	6.19†	1.36	5.91†	
22˚C Col +/+ 2met**		266.7 ± 100.4	40.2 ± 8.61	175.3 ± 48.9	20
22˚C Col *inv/inv* 2met**		243.2 ± 54.0	38.5 ± 11.9	197.2 ± 39.1	30
	t-value	1.07	0.552	-1.75	
22˚C Col +/+ 3met**		232.1 ± 54.4	31.2 ± 9.03	178.1 ± 54.6	30
22˚C Col *inv/inv* 3met**		245.9 ± 56.5	31.7 ± 7.96	159.8 ± 43.7	20
	t-value	-0.803	-0.209	1.25	
24˚C *acl1-1* +/+ 2met**		112.6 ± 62.3	28.6 ± 8.71	92.7 ± 35.5	40
24˚C *acl1-1 inv/inv* 2met**		25.6 ± 7.24	36.4 ± 10.9	72.1 ± 39.3	20
	t-value	6.20†	-2.99†	2.04†	
24˚C *acl1-1* +/+ 3met**		205.0 ± 83.1	31.5 ± 9.56	128.1 ± 31.2	30
24˚C *acl1-1 inv/inv* 3met**		23.2± 14.4	13.7 ± 6.30	37.4 ± 18.6	30
	t-value	11.81†	8.53†	13.67†	

† Difference is significant (significance level: $\alpha = 0.05$).

* Total number of cells assayed for calculate t-value.

** Metameric type of apical meristems: 2met, the type 2 metamer, main inflorescence stem differentiates cauline leaves with elongating internodes; 3met, the type 3 metamer, main florescence stem bears flowers without bracts formed upon the type 2 metamer (Schultz and Haughn, 1991).

Table I-4. Measurement of plant height at 26˚C

Genotype	Average ± standard deviation (cm)			t-value
acl1-1 +/+*	227.6	± 27.5	(n = 12)	t = - 0.350†
*acl1-1 inv/inv**	227.3	± 52.1	(n = 8)	t = - 0.242 †
Col +/+*	221.4	± 54.5	(n = 12)	
Col *inv/inv**	231.0	± 58.7	(n = 16)	t = - 0.519†

Plants were grown for 40 days.

*Length of the inflorescence stem was measured as plant height.

†Height of the plants is not significantly deviated from Col +/+.

Table I-5. Frequency of recombination between *acl1-3* and molecular markers on chromosome 4

Marker	Kb	Marker segregation in F2 Col/Col : Col/L*er* : L*er*/L*er*	Recombination frequency (%)
LD	1,124	8 : 5 : 2	30.0
nga8	5,629	54 : 25 : 1	20.8
FCA312	8,814	172 : 14 : 3	15.4
SC5	9,165	61 : 4 : 0	3.1
AG	10,384	296 : 6 : 0	2.0
CIW7	11,524	216 : 8 : 0	2.3
CAT2	16,701	89 : 4 : 0	3.1
DHS1	18,096	14 : 2 : 0	7.1

Table I-6. Segregation rate of the *acl1-1* phenotype in the F2 generation

	Phenotype		
Cross	Wild type	*acl1-1* (percentage)	χ^2
acl1-1 X Col	213	63 (22.8 %)	0.695 (P > 0.1)*
acl1-1 X L*er*	376	63 (14.4 %)	26.5 (P < 0.001)*

* Chi-square value for the expected ratio of 3 wild-type : 1 *acl1-1*.

Table I-7. Additional mapping markers generated for map-based cloning

Marker name (Kb)	Primer sequences (5'-3')	Restriction enzyme	Fragments (bp) Col	Fragments (bp) L*er*
PERL0770186 (9,253.4)	GTAGGATATGATCCTCTTTTGG GTTGACAGAAATGTCGAAAAGGGAT	*Bam*HI	128, 25	153
PERL0771474 (9,357.9)	CTATAAATACCCTGACTTGC TGAGAATGCGAATGGAACTAAGCAAAGAC	*Hin*fI	155	125, 30
PERL 0772901 (9,443.2)	TTCACCAGACTCTCTATTC TTTGTACTCGGTAGTACTCCATCGA	*Cla*I	143	118, 25
PERL0775439 (9,552.2)	CTTTGTTATACCTCATAATGGG TTATGTTCCTGGGAAATACAAGATC	*Bgl*II	125, 25	150
PERL0776597 (9,601.7)	CTCTTAGATGTAAGATTGTG TCGTCCTCTTCAGGTACCTGAGATC	*Bgl*II	121, 25	146
PERL0778995 (9,758.4)	GTACCTCTGTTTAGTGTG ATTACTACATGCCCCAAGATGATAT	*Eco*RV	125, 25	150

Table I-8. Composition of MS and MGRL media used in this study

MS (Wako)		MGRL	
Concentration (mM)		Concentration (mM)	
NH_4NO_3	20.6	Na_2HPO_4	1.5
KNO_3	18.8	NaH_2PO_4	0.26
$CaCl_2•2H_2O$	3.0	KCl	3.0
$MgSO_4•7H_2O$	1.5	$Ca(NO_3)_2•4H_2O$	5.0
KH_2PO_4	1.25	$MgSO_4•4H_2O$	1.5
H_3BO_3	0.10	NH_4Cl	0.50
$MnSO_4•4H_2O$	0.10	H_3BO_3	0.003
$ZnSO_4•7H_2O$	0.03	$MnSO_4•4H_2O$	0.010
KI	0.005	$ZnSO_4•7H_2O$	0.001
$Na_2MoO_4•2H_2O$	0.001	MoO_3	0.0002
$CuSO_4•5H_2O$	0.0001	$CuSO_4•5H_2O$	0.0001
$CoCl_2•6H_2O$	0.0001	$CoCl_2•6H_2O$	0.0001
Na_2-EDTA	0.11	Fe(III)-EDTA	0.02
$FeSO_4•7H_2O$	0.10		

Table I-9. Primers and restriction enzymes used for genotyping defense mutants

Genotype	Primers (5'-3')	Restriction enzyme
EDS1	ACACATCGGTGATGCGAGACA	
	GGCTTGTATCATCTTCTATCC	
eds1-2	ACACATCGGTGATGCGAGACA	
	GTGGAAACCAAATTTGACATTAG	
PAD4/*pad4-1*	GAAGCAGCAATGAACAATTC	*Fin*I digests wild-type product
	CACTCCTCAGGCACTTTAAC	
NDR1	TGGTTTAAGCATGAGAGTCC	
	TTCGACCACCTTCTGTGTC	
ndr1-1	CCAACTAAGCACATTTTGGG	
	CCCAACATATAATTGTTTCTTG	
rar1-21	GGAATGAAAGAGTGGAGCTGCTACTAG	*Spe*I digests mutant product
	TTTTGGAACCGATTTGGCCAG	
SID2	CAACCACCTGGTGCACCAGC	
	AAGCAAAATGTTTGAGTCAGCA	
sid2-2	TTCTTCATGCAGGGGAGGAG	
	AAGCAAAATGTTTGAGTCAGCA	
npr1-1	GTCTCGAATGTACATAAGGC	*Nla*III digests wild-type product
	ATCATGAGTGCGGTTCTACC	
SNC1	ATGACAAGTTGACATCGG	
	CCTGAATGAATTGGTGGAGA	
snc1-11 (*NPTII*)	ATTGAACAAGATGGATTGCACG	
	TCAGAAGAACTCGTCAAGAAGG	
RPP4	ATCAATTTGCGTTGGCATCC	
	GGAGATTTGATTTTAGCCAC	
rpp4	ATCAATTTGCGTTGGCATCC	
	TTAGGCGACTTTTGAACGCG	

Table I-10. Gene function and sequence of primers used for expression analysis of genes located near and within the inversion

Locus	Function	Primers (5'-3'), forward and reverse
At4g21940	CALCIUM-DEPENDENT PROTEIN KINASE 15 (CPK15)	TGGACAAGAGAACATTGTTG
		CTCTGCAATAACCTTTAGAG
At4g21950	Unknown protein similar to AT4G04630.1	CGAAGAATATTCAATTAAAGAG
		M13 primer M4 (Takara)
At4g21970	Similar to Os05g0114600	GGAAGGGGGAGAAGAGGTTTC
		CTAAAGAGTTAAAAGACCATTG
At4g21980	AUTOPHAGY 8A (APG8A)	CTAAACCTCTCGAGGCAAG
		TCAAGCAACGGTAAGAGATC
At4g21990	APS REDUCTASE 3 (APR3)	ATTGTTGCTTCTGAGGTTAC
		CAACATTCTCGCTATTGAAG
At4g22000	Hypothetical protein	TGGAAAAGCTGCAGAAGCTG
		M13 primer M4 (Takara)
At4g22010	SKU5 SIMILAR 4 (SKS4)	TCGAACTATCCGGAGAAATC
		TGGATATTCATCTCGGTACG
At4g22030	F-box family protein	TAGCTTGTCTAGGTTTGATG
		AAAGATACAACAGACTTGAG
At4g22050	Aspartyl protease family protein	AATGTTCCAATGGATTCCGC
		ACTTAGCGAACCCAACTTTC
At4g22060	F-box family protein	AGCTTCCTTTAGATCTCTTG
		M13 primer M4 (Takara)
At4g22070	WRKY DNA-BINDING PROTEIN 31	TGAAGCTGCCATGATAAGCG
		ACACATCCGAAACTCAAAAC
At4g22080	Pectate lyase family protein	GAGAATGCCAAGAGTAAGAC
		AAGTTTCCCGGAGCTACTG
At4g22090	Pectate lyase family protein	AGAATGCCAAGGGTAAGACG
		TCCGGGAGCGACTGTGAATC
At4g22100	Glycosyl hydrolase family 1 protein	TCCTTCACACTCGTAACC
		GAAATCTTTGGCTCTTTGAAC

At4g22105	SCR-LIKE 26 (SCRL26)	GCTACTTTTTTCTTGGTTTC
		TACACCGGCATAAATGTTCCG
At4g22110	Alcohol dehydrogenase	AAAAATTCGAGCTAGGCAAG
		AAAGAATGCAGCGGAGAGAC
At4g22115	SCR-LIKE 14 (SCR L14)	GGGCAATGTTAAAGAAGTGG
		GCAAGGAACATAACATCTAC
At4g22120	Early-responsive to dehydration protein-related protein	GAATTATGGTGAAGCTTGGC
		CGGTTTCTAATGTATCTTTC
At4g22130	STRUBBELIG-RECEPTOR FAMILY 8 (SRF8)	ACTGAGAGACAGGTTTCAAC
		CAGAATGAGATATCGACGTG
At4g22140	DNA binding protein	CAAAGTTGTGAGAGCGGGAG
		AAACGGACCTTAACATCATC
At4g22150	LANT UBX DOMAIN-CONTAINING PROTEIN 3	CTTCTTTTCTTGATAGCATTC
	(PUX3)	TGAATGACTACAGAACTTGC
At4g22160	Unknown protein	AGAATGTCTTGTTGGGTAAAG
		M13 primer M4 (Takara)
At4g22165	F-box family protein	AACATAACCCTAATTCCTGG
		TACGAACCAATGAGCTCTAG
At4g22170	F-box family protein	AACATAACCCTAATTCCTGG
		TACGAACCAATGAGCTCTAG
At4g22180	F-box family protein	AAGCGTCTCAGAGGAGATAC
		TAACATATGAAGAAGAAAGTTC
At4g22190	similar to conserved hypothetical protein	GTGTTATGTACCACAAACTC
		M13 primer M4 (Takara)
At4g22200	ARABIDOPSIS K+ TRANSPORTER (AKT2/3)	ATTTGGAACGTTTCTTACCC
		ACATCCACATAAGAGATGTG
At4g22210	LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 85	ATGTCTCCTACAGATGGGC
	(LCR85)	TCACATGCTTTCCATTTCAG*
At4g22212	Defensin-like (DEFL) family protein	CATATCTCCTACAGAAGTAG†
		TCACATGCTTTCCATTTCAG*
At4g22214	Defensin-like (DEFL) family protein	ATCTCCTACAGAAGCAGTG
		ATGCCTGCTTTTTTATATCC
At4g22217	Defensin-like (DEFL) family protein	CTATCTCCCACCGAAGTGG

		TATTGAGCACGGATACTATC
At4g22220	IRON-SULFUR CLUSTER ASSEMBLY COMPLEX	TTCTTCATCTGTTGCCACTG
	PROTEIN (ISU1)	CACCATTTGTCTTCACACG
At4g22230	Defensin-like (DEFL) family protein	TTTCCTTTTCAGCACCACGC
		CATATCTCCTACAGAAGTAG†
At4g22233	Potential natural antisense gene	CTGATCGAAGTCGCTAAC
		TATGATCCATACAAAGAGAC
At4g22235	Defensin-like (DEFL) family protein	TCTCCTATAGAAGTGAATGG
		ACATGCTTTCCTTTTCAGC
At4g22240	Plastid-lipid associated protein	AAGTAAACCTACAACCACAC
		AAATGGATCCTCGCCTACAC
At4g22260	IMMUTANS (IM)	GCTCATAATGGAAGAATTGG
		GCAACCACAAAGGCTAGTAG

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*. †, The same primers were used because these genes shared highly similar sequences.

Table I-11. Primers used for semi-quantitative RT-PCR

Gene	Primer 1 (5'-3')	Primer 2 (5'-3')
EDS1	TGCTC GATCA CCTGA ATAAT C	ACACA TCAAC TGTTG CAAAC
PAD4	CAGTT AAAGA TCAAG GAAGG	TGTAG AAATT CGCAA TGTCG
NDR1	ATGAA GACAC AGAAG GTGG	CGAAT AGCAA AGAAT ACGAG
SID1	CTGGT CGCAG AATCG GTG	GCCGA AACAA TCTGT GAAG
SID2	CCATC TCTCG TAGTT ACTC	CATTA AACTC AACCT GAGGG
NPR1	GATCT TGAAA ATAGA GTTGC AC	ACGAT GAGAG AGTTT ACGG
PR1	GAATT TTACT GGCTA TTCTC TG	TTAGT ATGGC TTCTC GTTC
PR2	TTCAA CCACA CAGCT GGAC	ACTTA GACTG TCGAT CTGG
PR5	CTCGT GTTCA TCACA AGC	AGGGC AGAAA GTGAT TTC
PDF1.2	TGCTT TCGAC GCACC GG	CTCAT AGAGT GACAG AGAC
RPP4	AATAC GTGTG CCACC CAC	CTCGA TCTCA TTTCT ATCTT G
At4g16880	GTATG TTACC AAAGA TTTCA AG	CGTAT GAATT ACCTG GACG
SNC1	CTTCA TAGAT TGGTG AAGTT AG	TCAGT TACCA GAAAC AGGAA AC
At4g16900	CTGTA GGGCA GGTGG AG	TTAAC GTATT CTAGA ATCC
At4g16920	GCGGA TGGGG ATGAC AT	TCAGT TACCA GAATC AGTAG
At4g16930	GATTC TCGAA ACTGG TGTAA TG	CCTGT TCTTC TCGGT TGG
At4g16940	ACGTT TAACA CCGAA TG	CATCA CAGCG TTGAG TCTTC
At4g16950	CTCTT TTTTG CCCCT TCTTC	GATCT TCTGA ACGGG CCTAA TG
At4g16960	ACGTT TAACA CCGAA TG	TTCCC AAGGG ACTGG AC
At4g16990	GATTG CCGGT AATCG TC	CCTTG TTCAC AGTAC TC
SRFR1	ATGGC GACGG CGACG GC	CTGTG TTCGC CTAAT CCATG
EF1a	ACTTG CAGCT ATGGG TAAAG	CGAAA GTCTC ATCAT TTGGC
SRFR1 for cDNA sequencing	ATGGC GACGG CGACG GC	TCAAT CGTTG TAAGT GCTAA G

インターネット公表に関する使用承認が雑誌社（出版社）から得られていないため、
本図については、非公開。
「An inversion identified in *acl1-1* mutant functions as an enhancer of the *acl1-1* phenotype」
Genes and Genetic Systems 誌 83巻、293~300頁
Fig. 1 参照

インターネット公表に関する使用承認が雑誌社（出版社）から得られていないため、
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「An inversion identified in *acl1-1* mutant functions as an enhancer of the *acl1-1* phenotype」
Genes and Genetic Systems 誌 83巻、293~300頁
Supplementary Fig. 1 参照

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「An inversion identified in *acl1-1* mutant functions as an enhancer of the *acl1-1* phenotype」
Genes and Genetic Systems 誌 83巻、293~300頁
Fig. 2 参照

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「An inversion identified in *acl1-1* mutant functions as an enhancer of the *acl1-1* phenotype」
Genes and Genetic Systems 誌 83巻、293~300頁
Fig. 3 参照

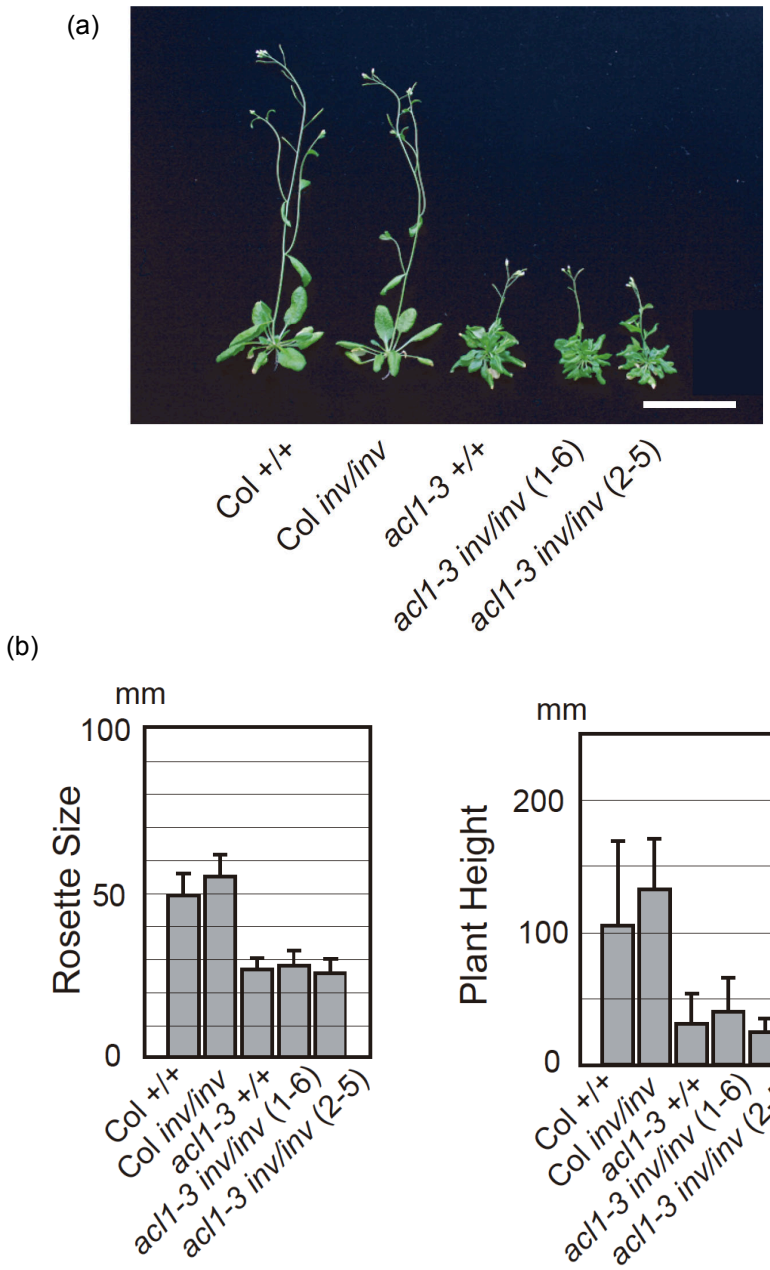

Figure I-5. Comparison of plant growth between Col *+/+* and *Col inv/inv*, and *acl1-3 +/+* and *acl1-3 inv/inv.*

(a) Overall appearance of Col +/+, Col *inv/inv*, *acl1-3* +/+ and two plants from distinct lines of *acl1-3 inv/inv*. Number in the parenthesis indicate the line number. Bar = 5 cm.

(b) Rosette size and plant height of plants of Col +/+, Col *inv/inv*, *acl1-3* +/+ and two *acl1-3 inv/inv* lines. Error bars indicate standard deviation.

Plants were grown for 40 days at 22˚C.

5年以内に雑誌等で刊行予定のため、本図については非公開。

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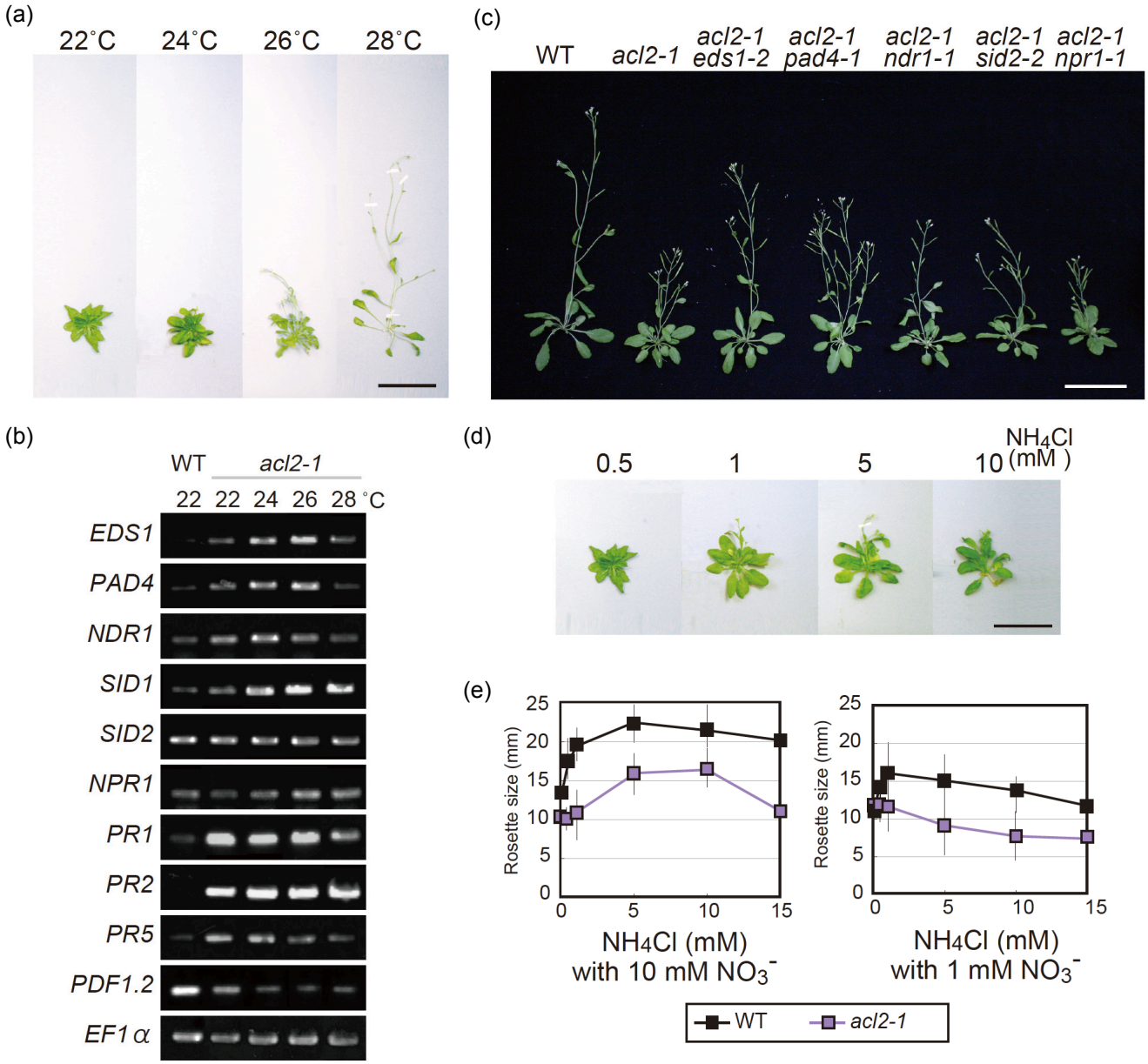


Figure I-14. Phenotype of the *acl2-1* mutant

(a) Temperature-dependent growth of the *acl2-1* mutant. Plants were grown at indicated temperature for 40 days, and watered with MGRL medium.

(b) Semi-quantitative RT-PCR of the defense-related genes in the *acl2-1* mutant. *EF1α* was used as control.

(c) Double mutants between *acl2-1* and mutants of key regulatory genes in defense responses. Plants were grown for 40 days at 22˚C, and watered with MGRL medium.

(d) Growth of *acl2-1* mutant watered with the nitrogen-free medium supplemented with 10 mM nitrate and indicated concentrations of NH_4Cl.

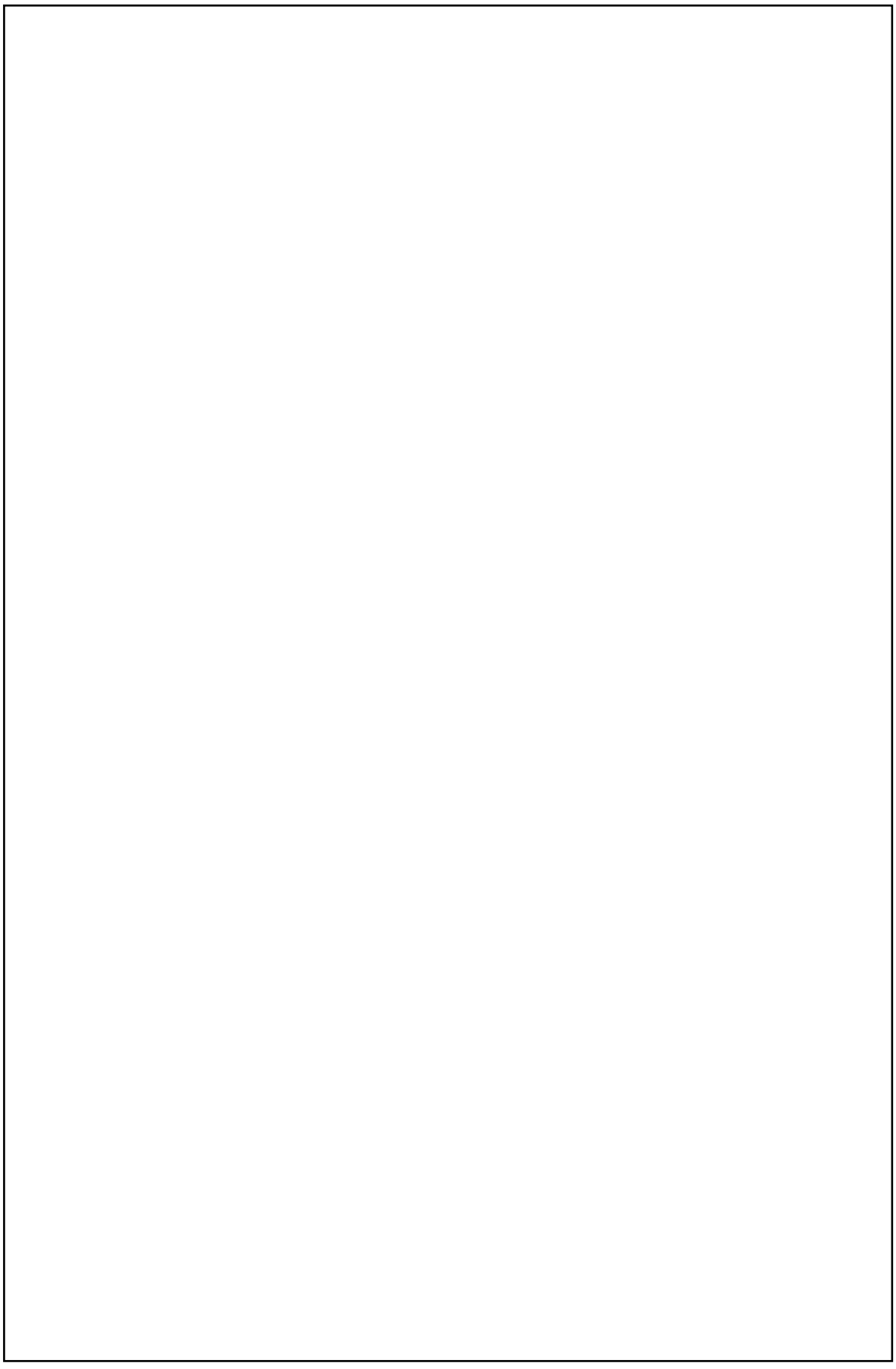
(e) Growth of *acl2-1* seedlings on the media containing different concentrations of NO_3^- and NH_4Cl.

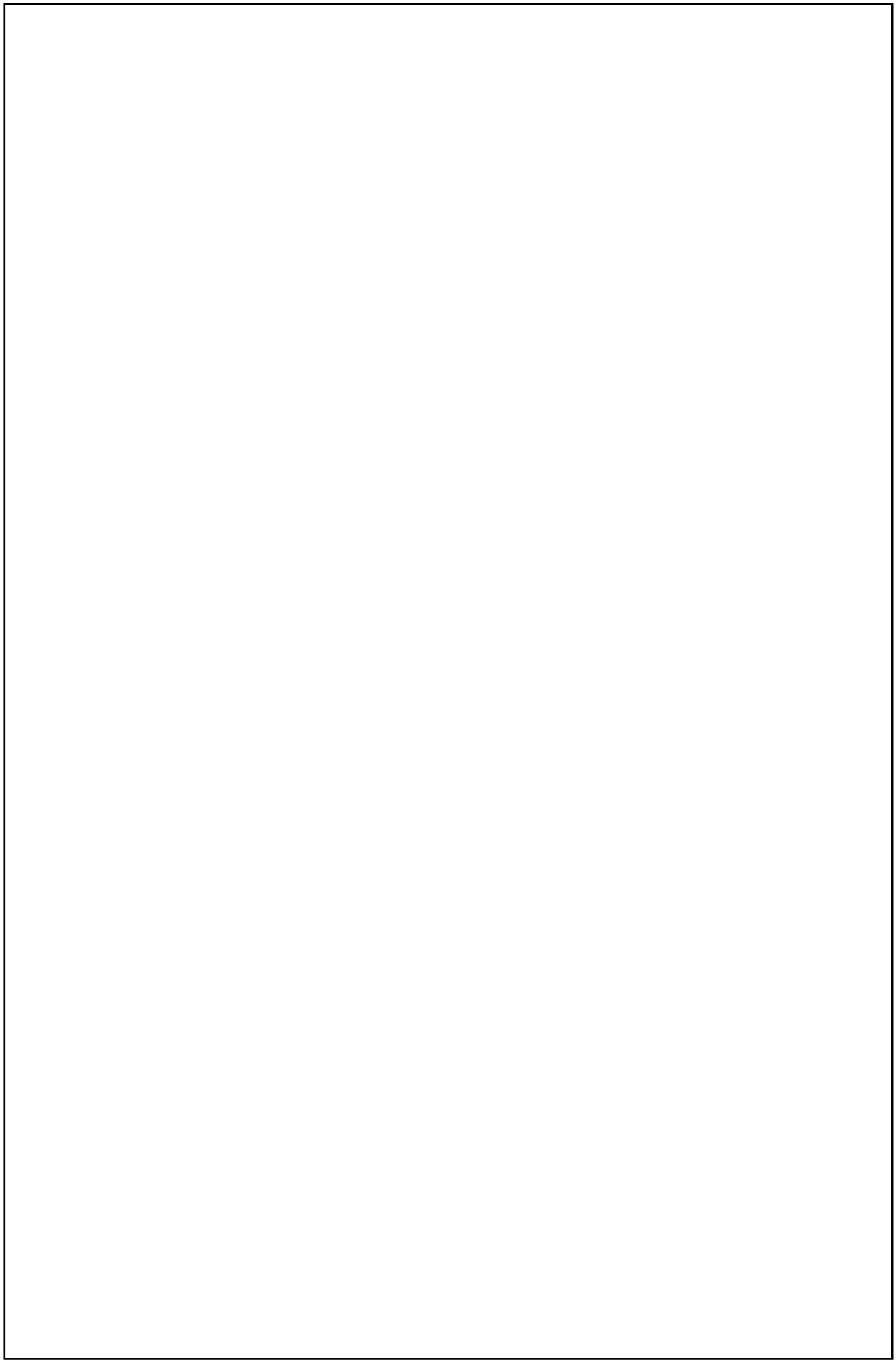
Bars = 5 cm.

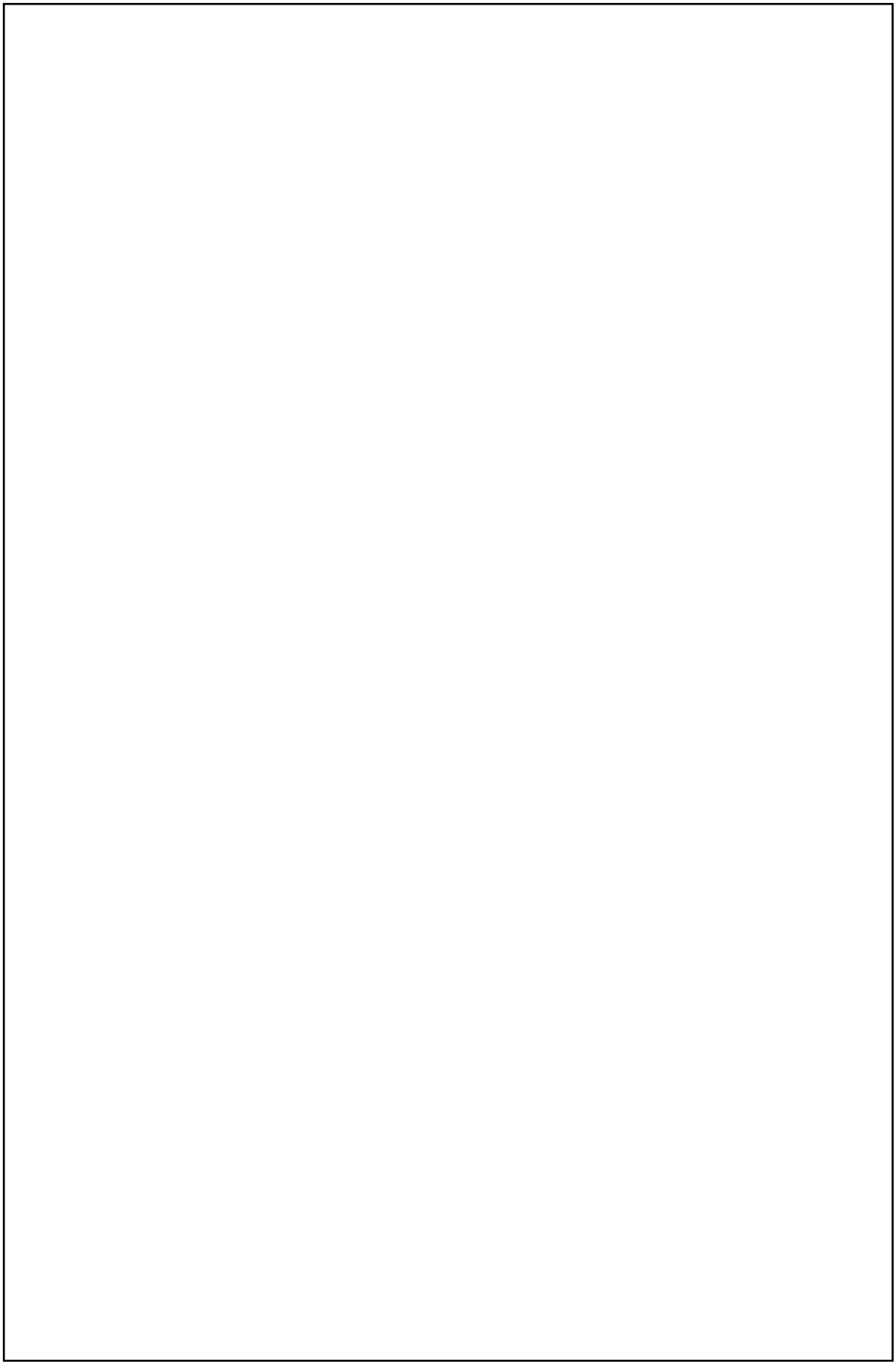
Chapter II: Mutations in epidermis-specific HD-ZIP IV genes affect floral organ identity in *Arabidopsis thaliana*.

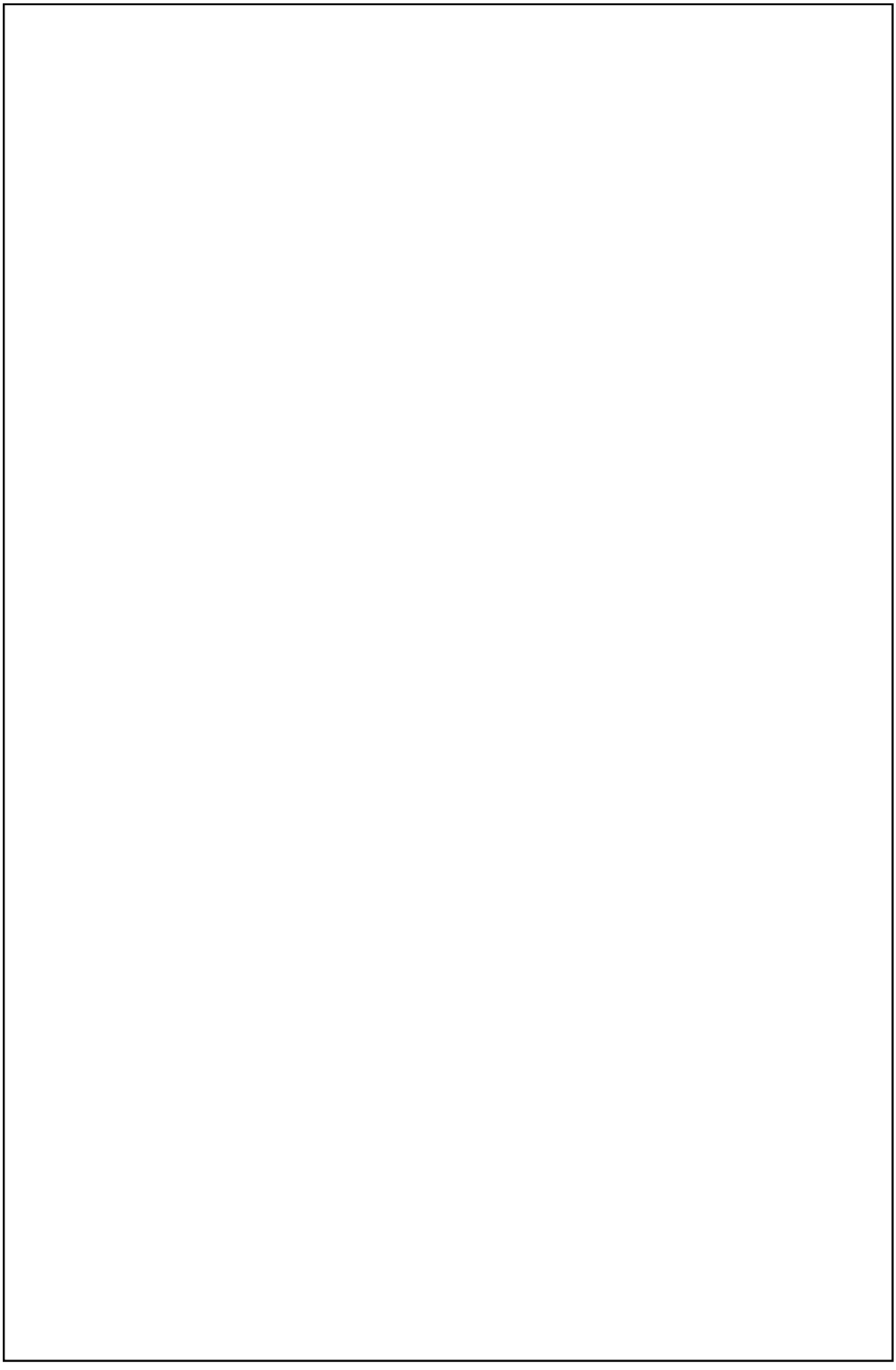
インターネット公表に関する使用承認が雑誌社（出版社）から得られていないため、
本章については、非公開。

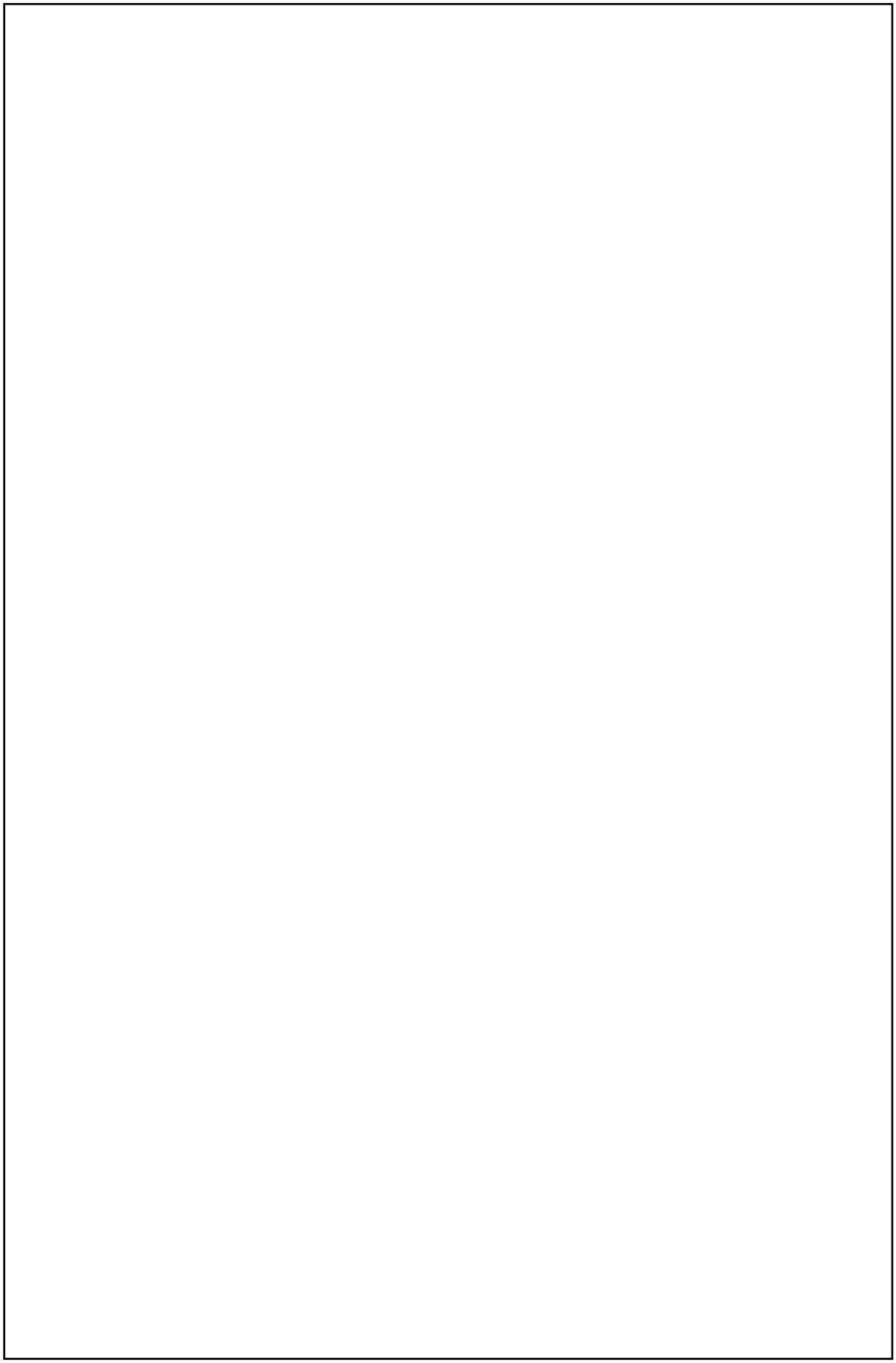
「Mutations in epidermis-specific HD-ZIP IV genes affect floral organ identity in *Arabidopsis thaliana*」
*Plant Journal*誌　75巻　430~440頁

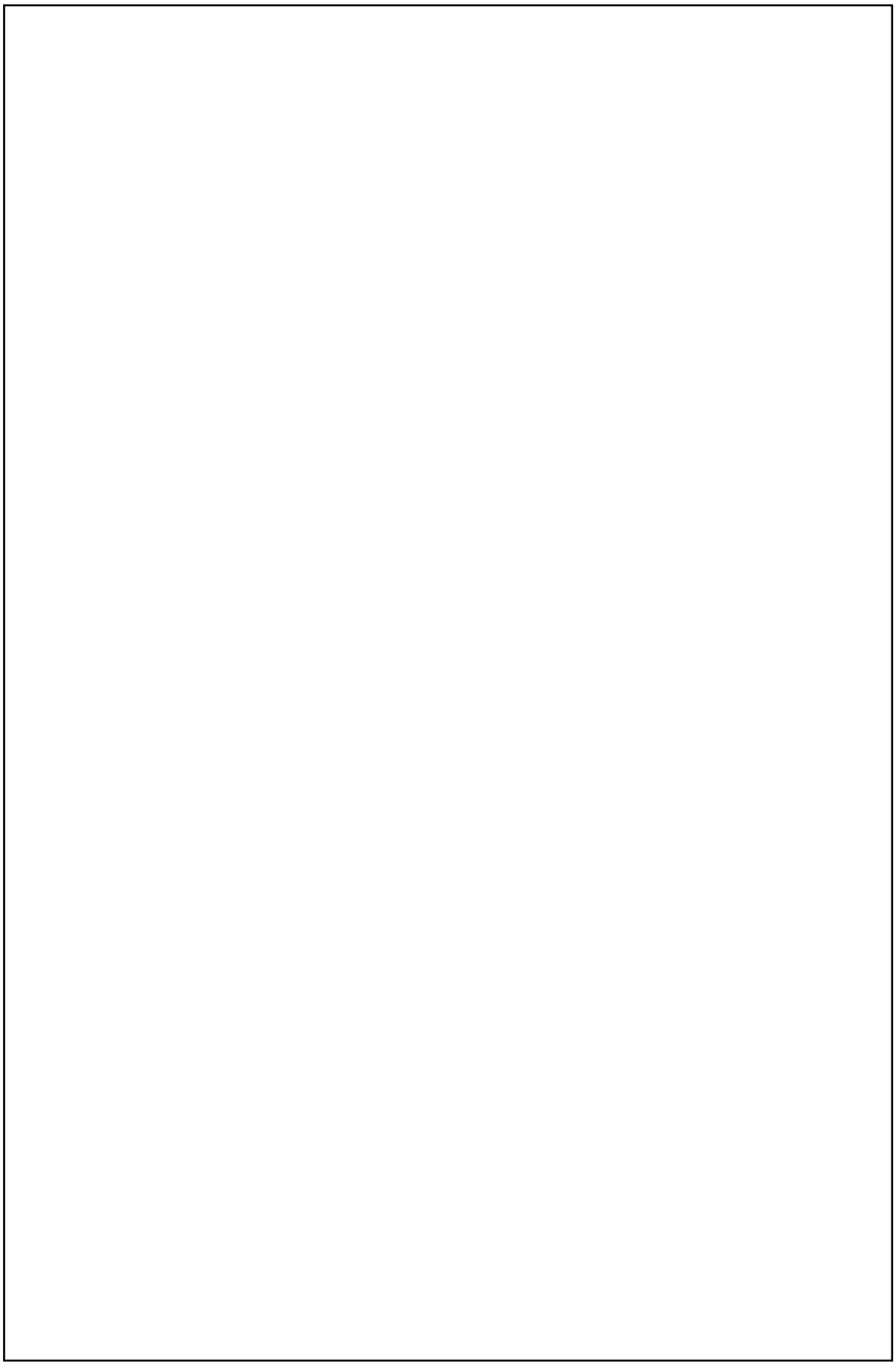


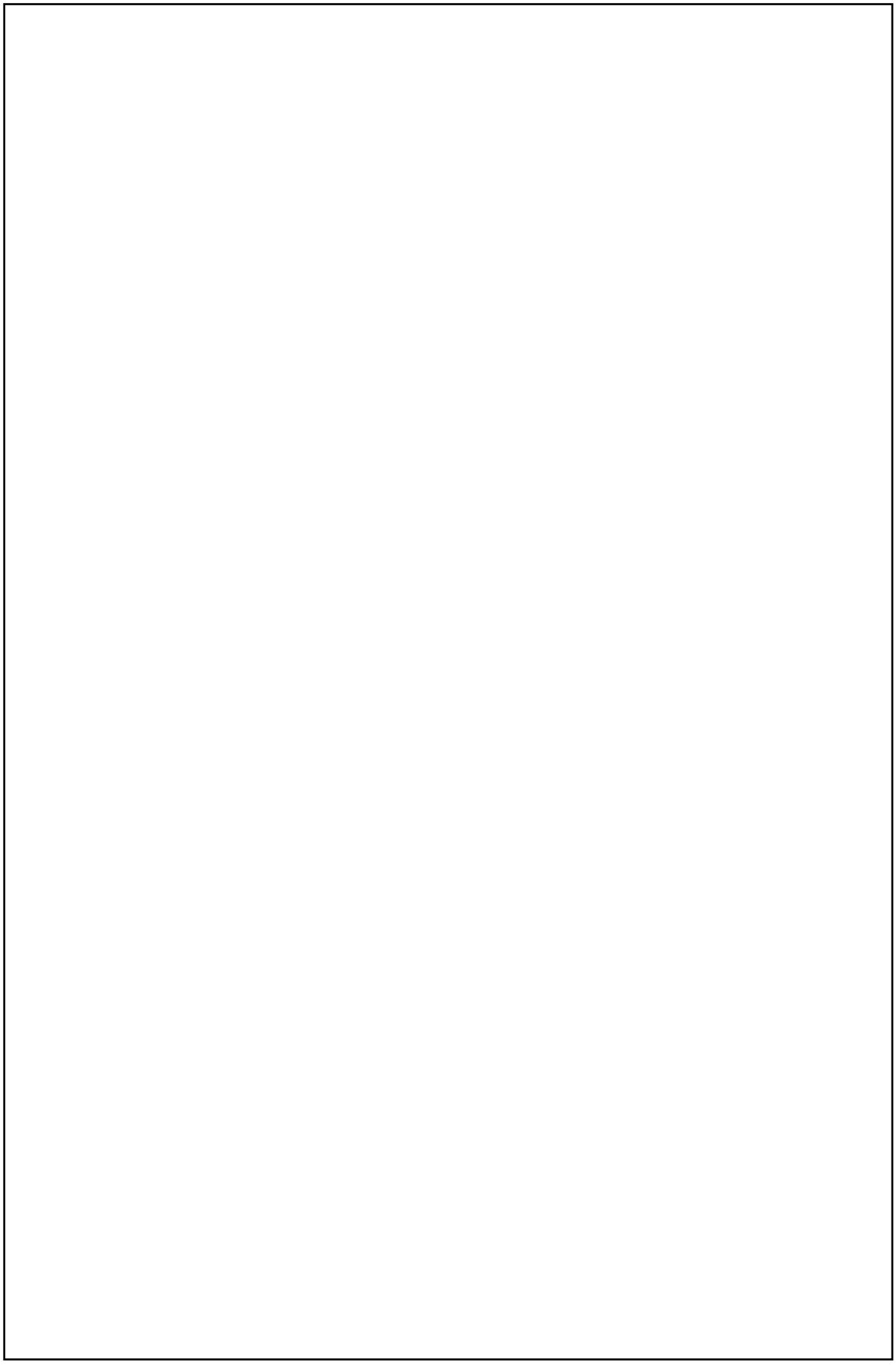


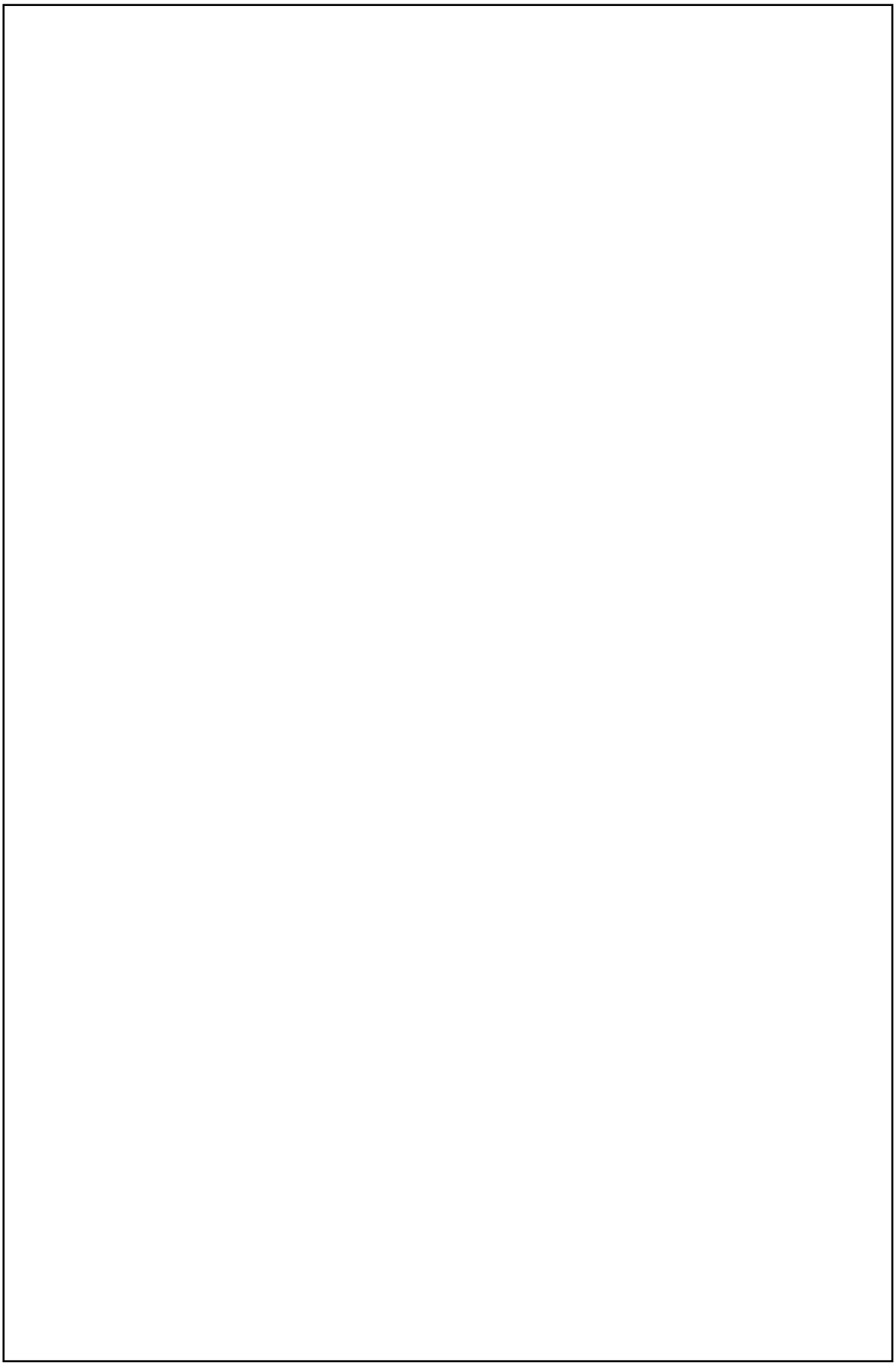


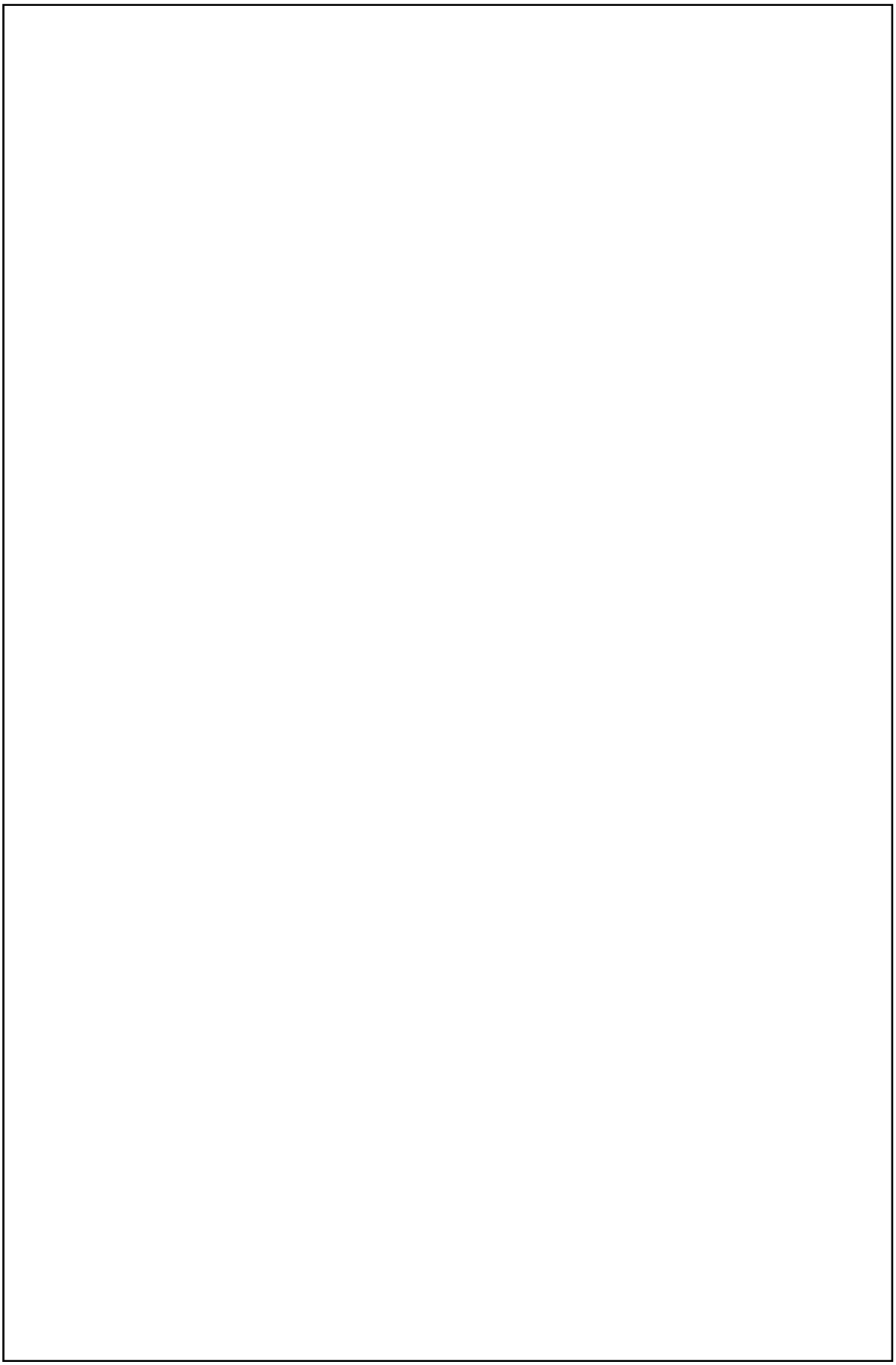


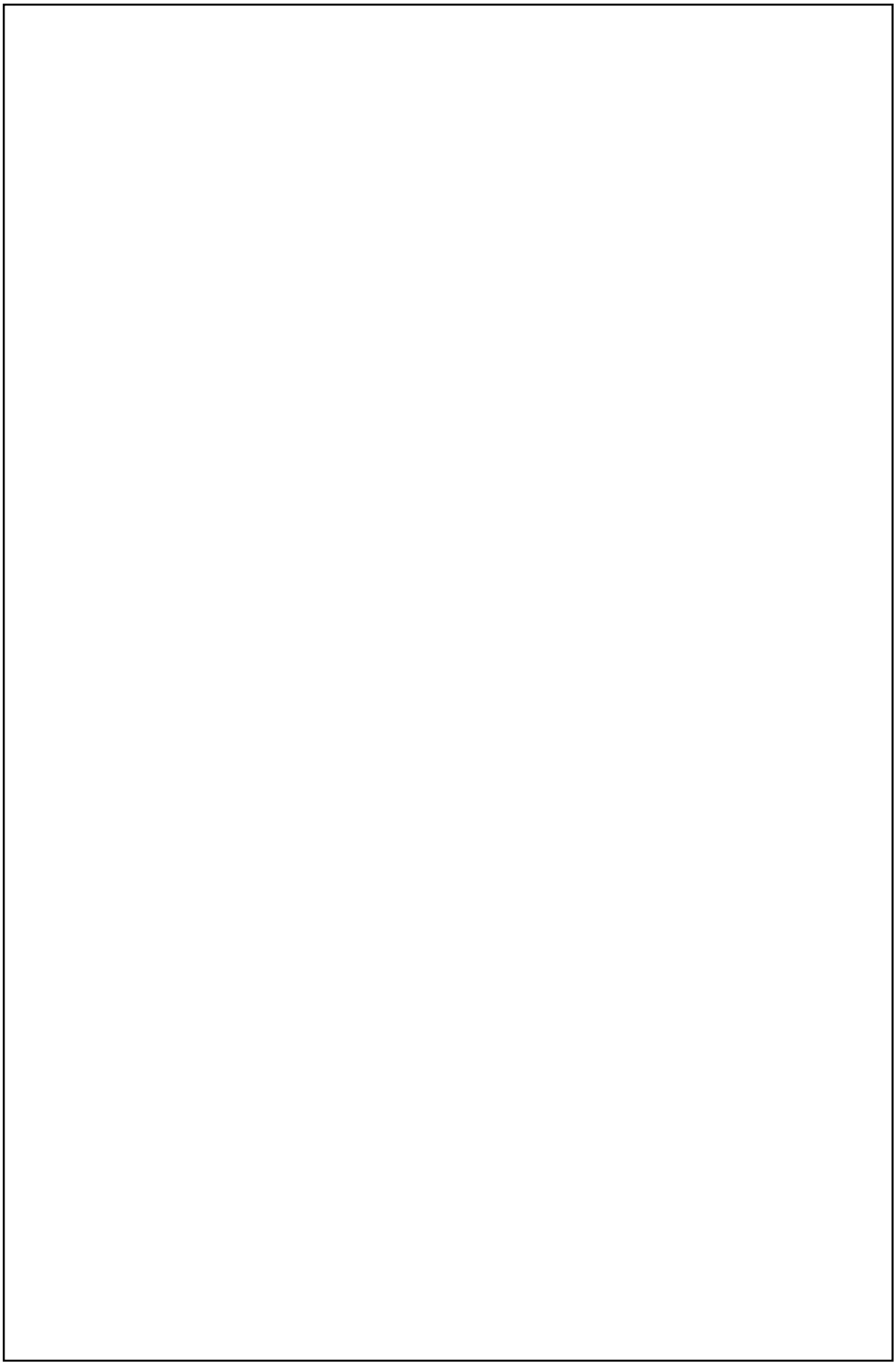


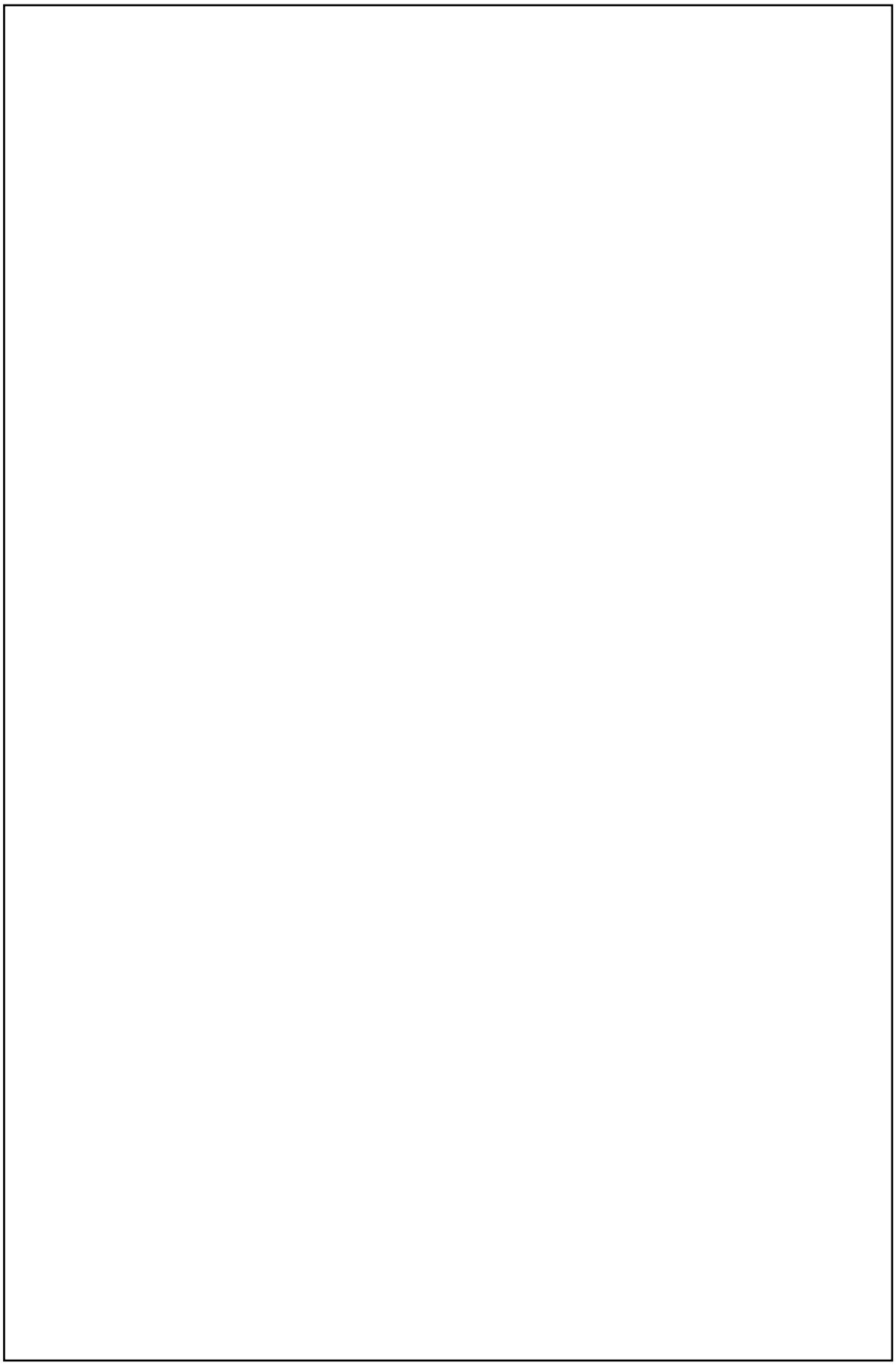


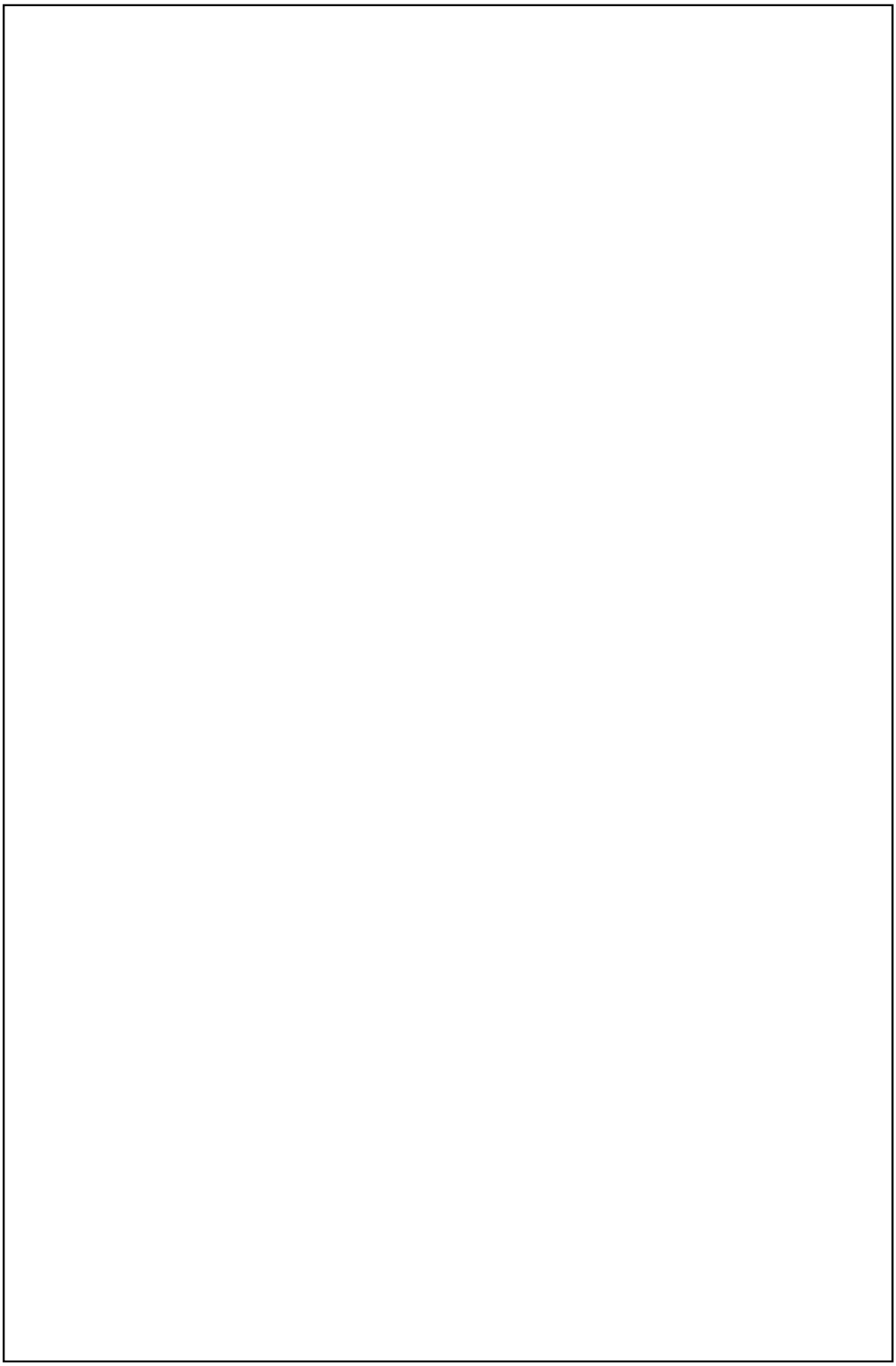


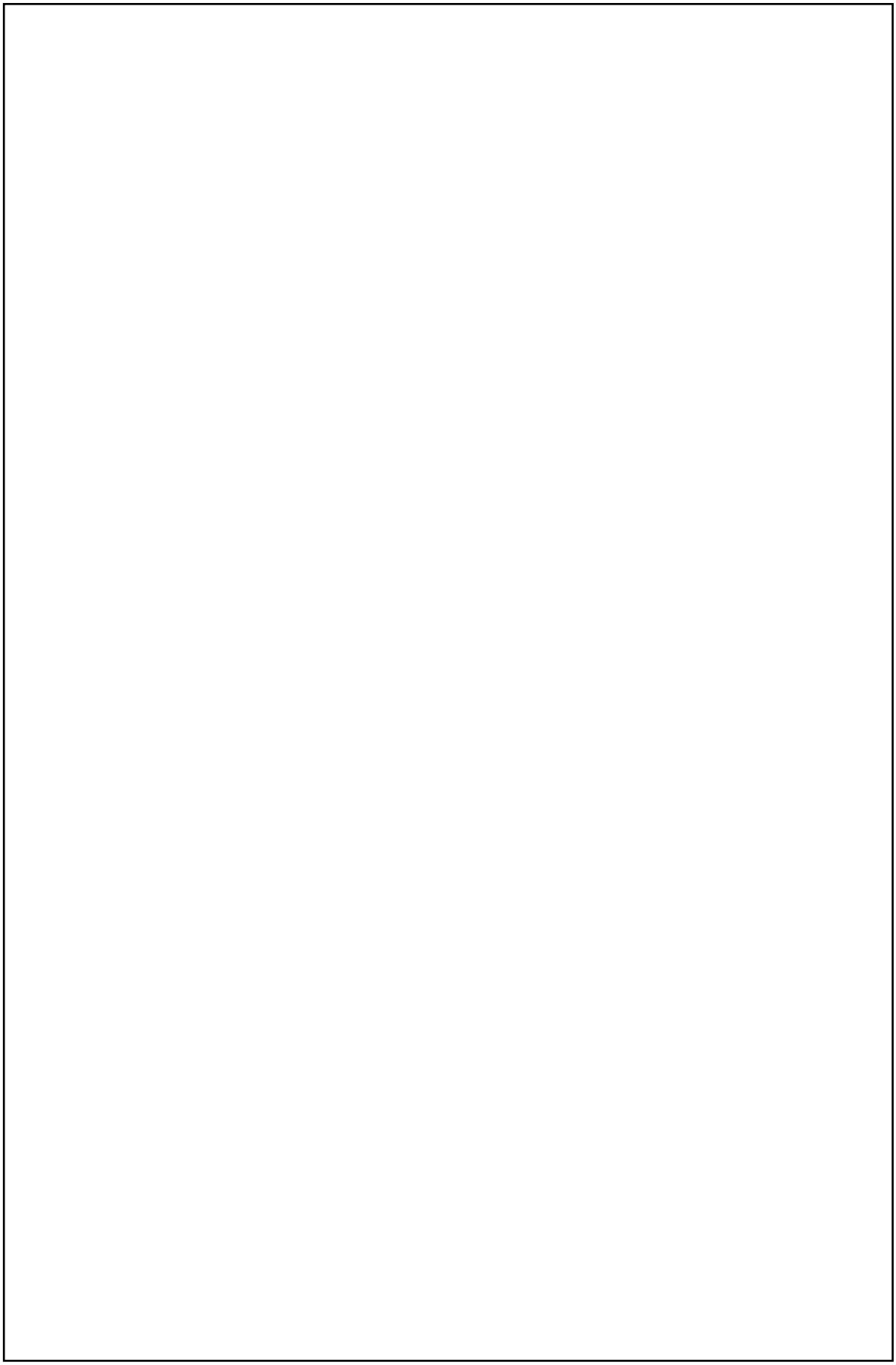


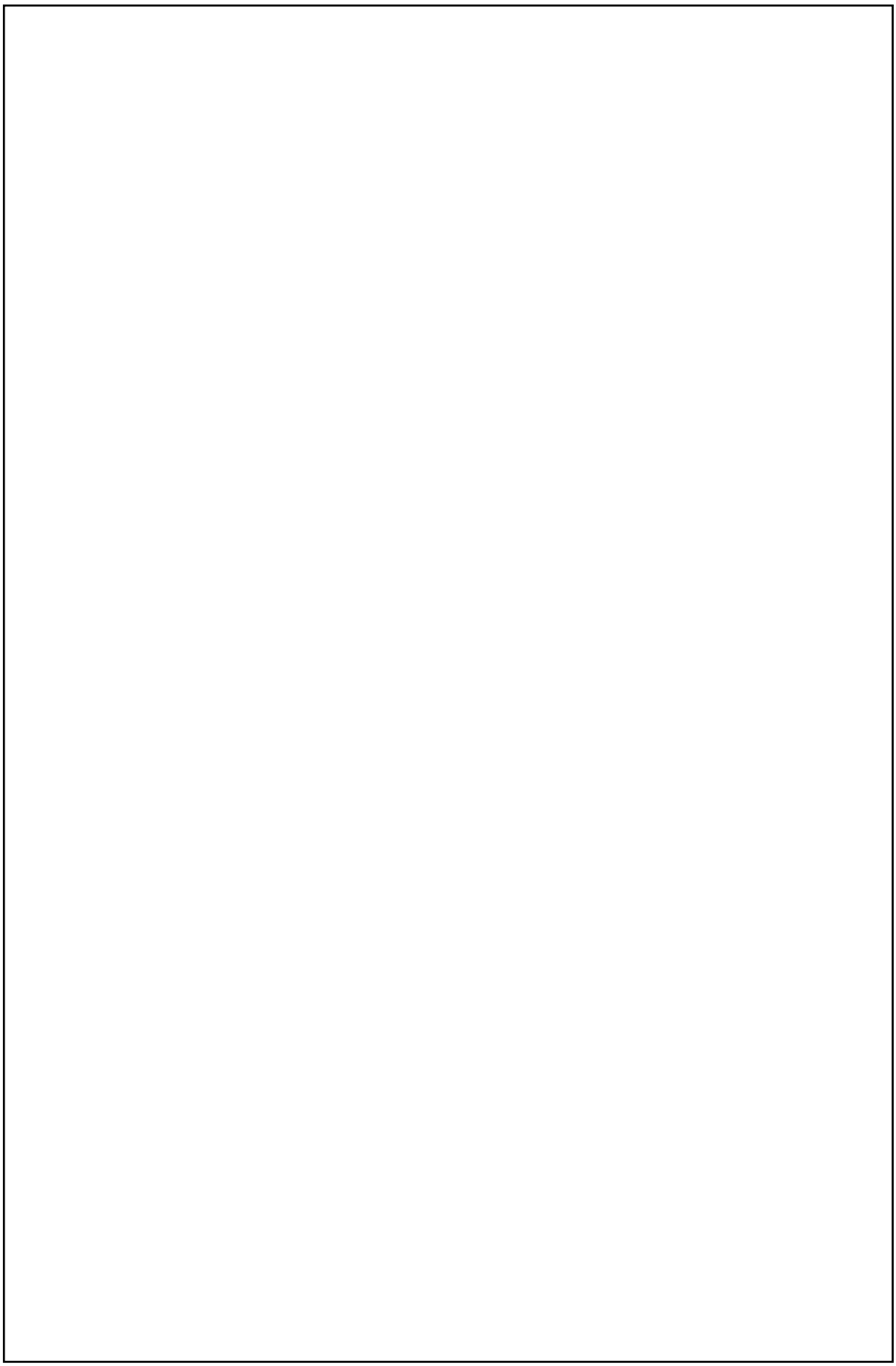


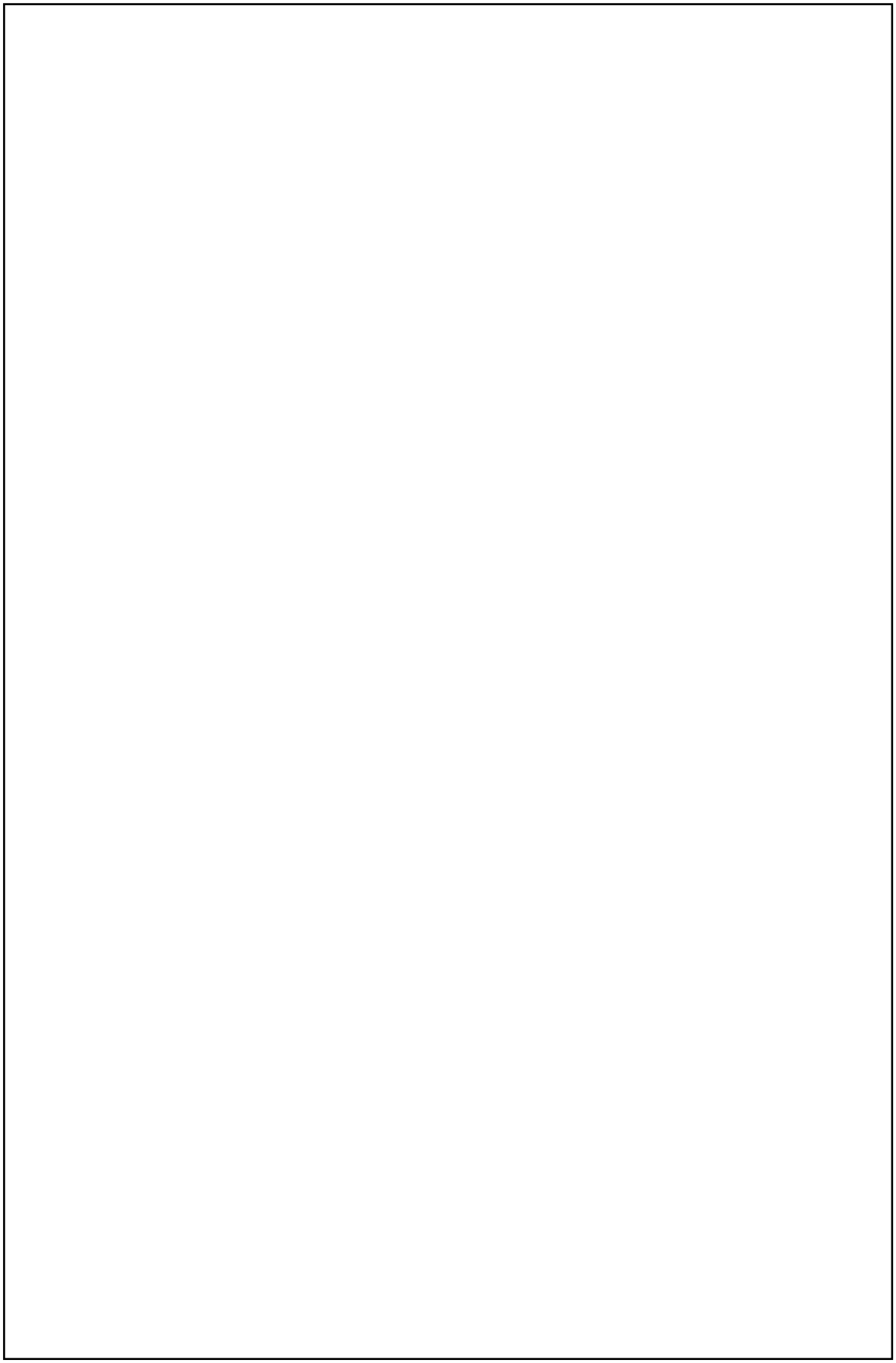


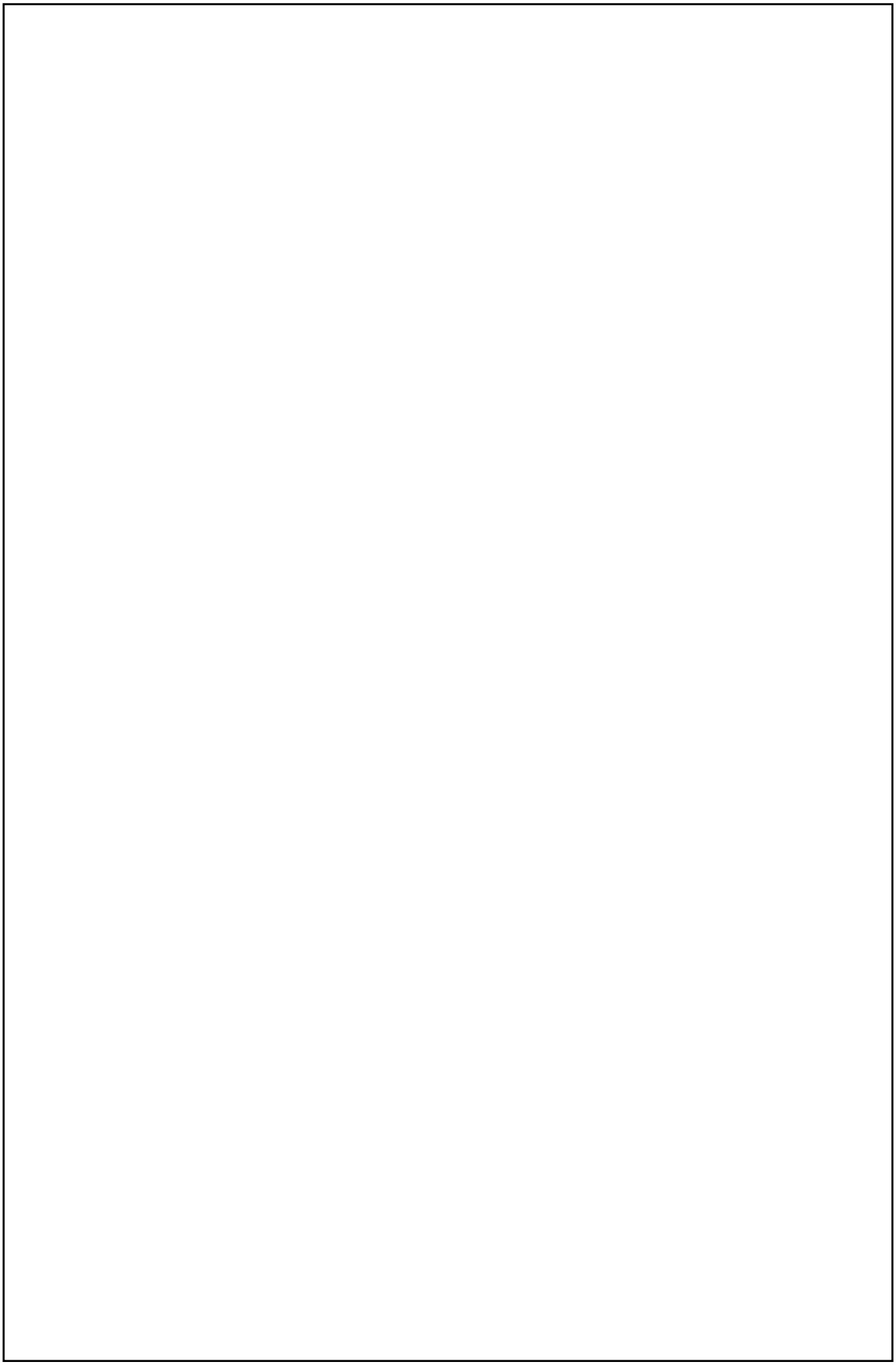


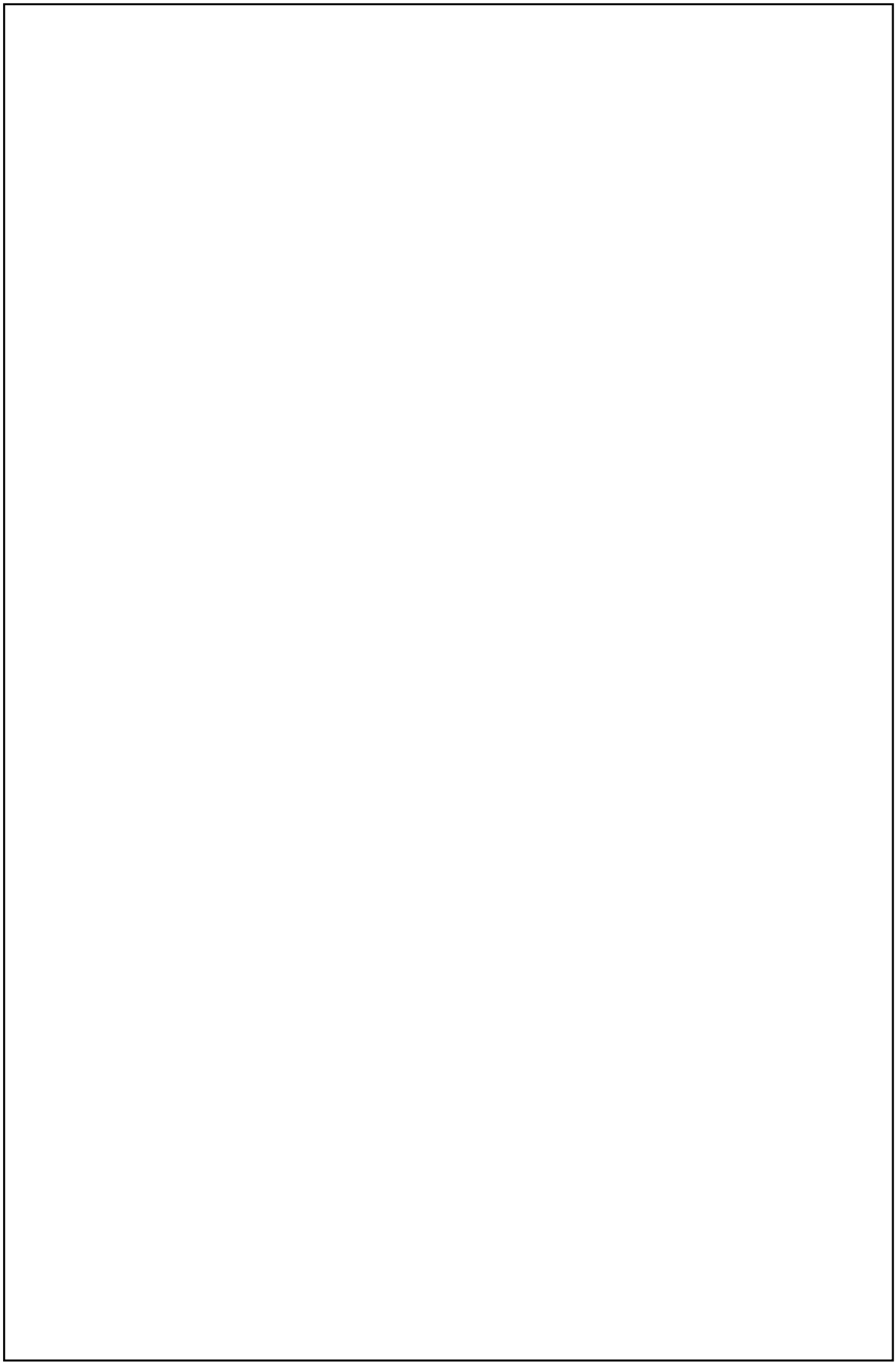


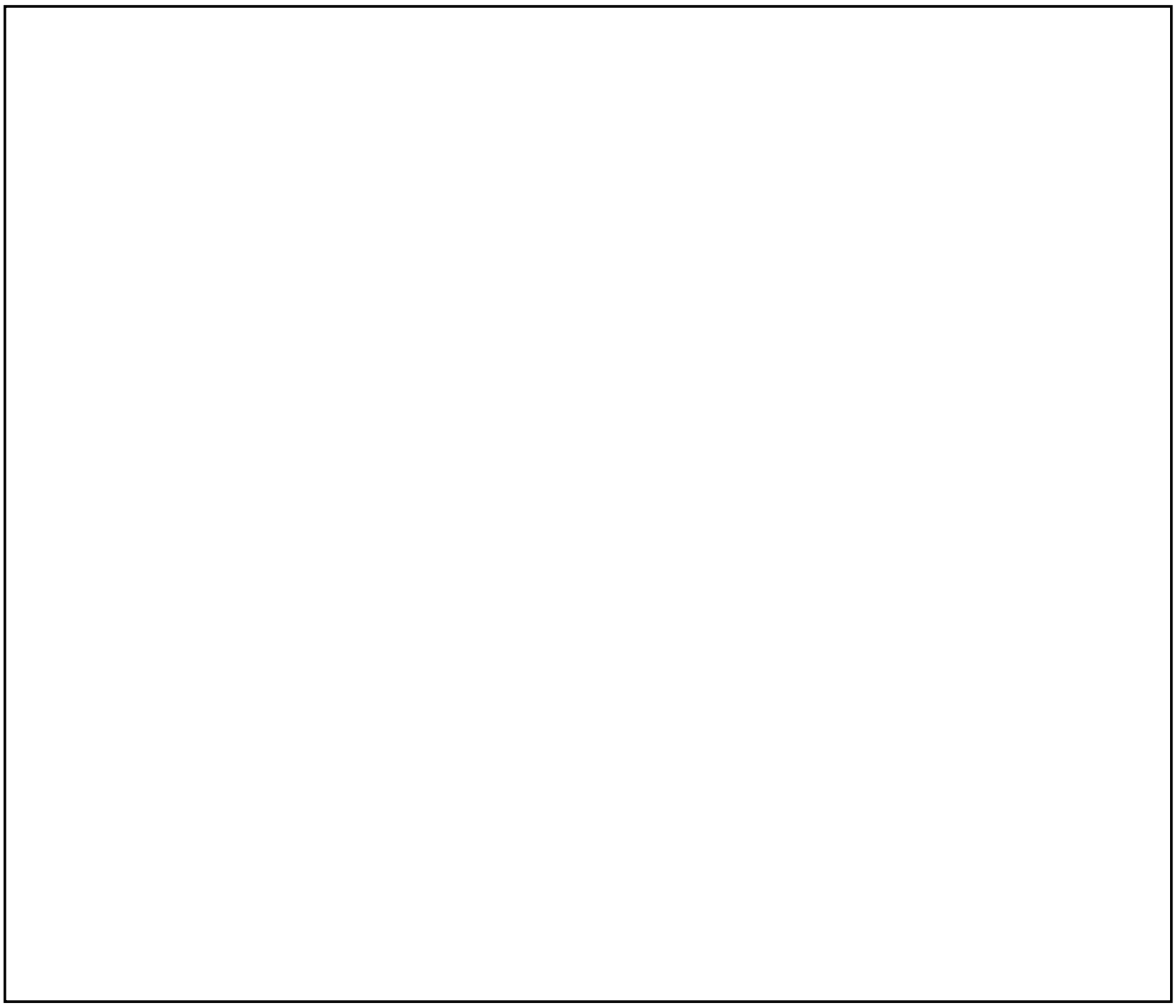


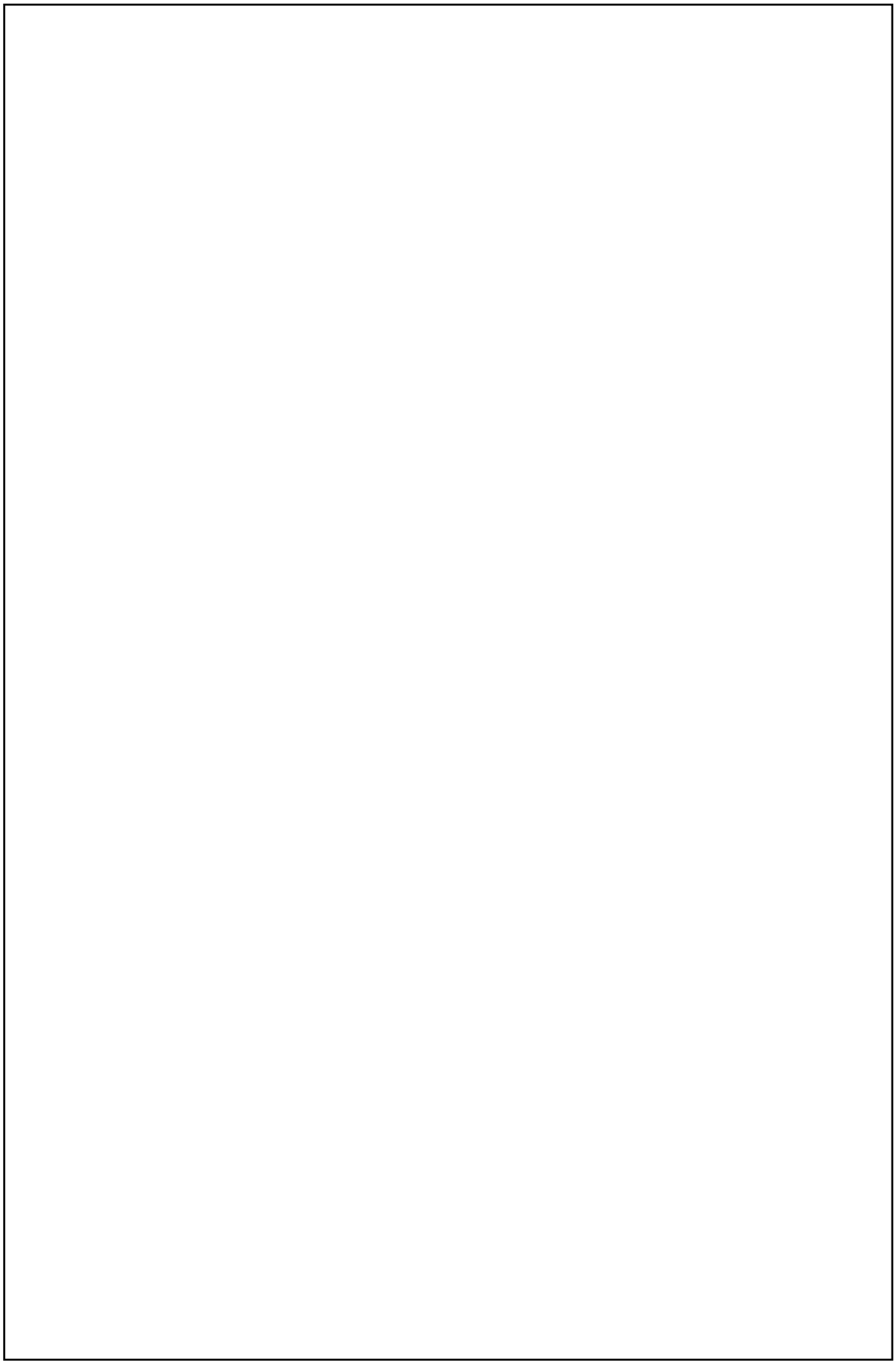


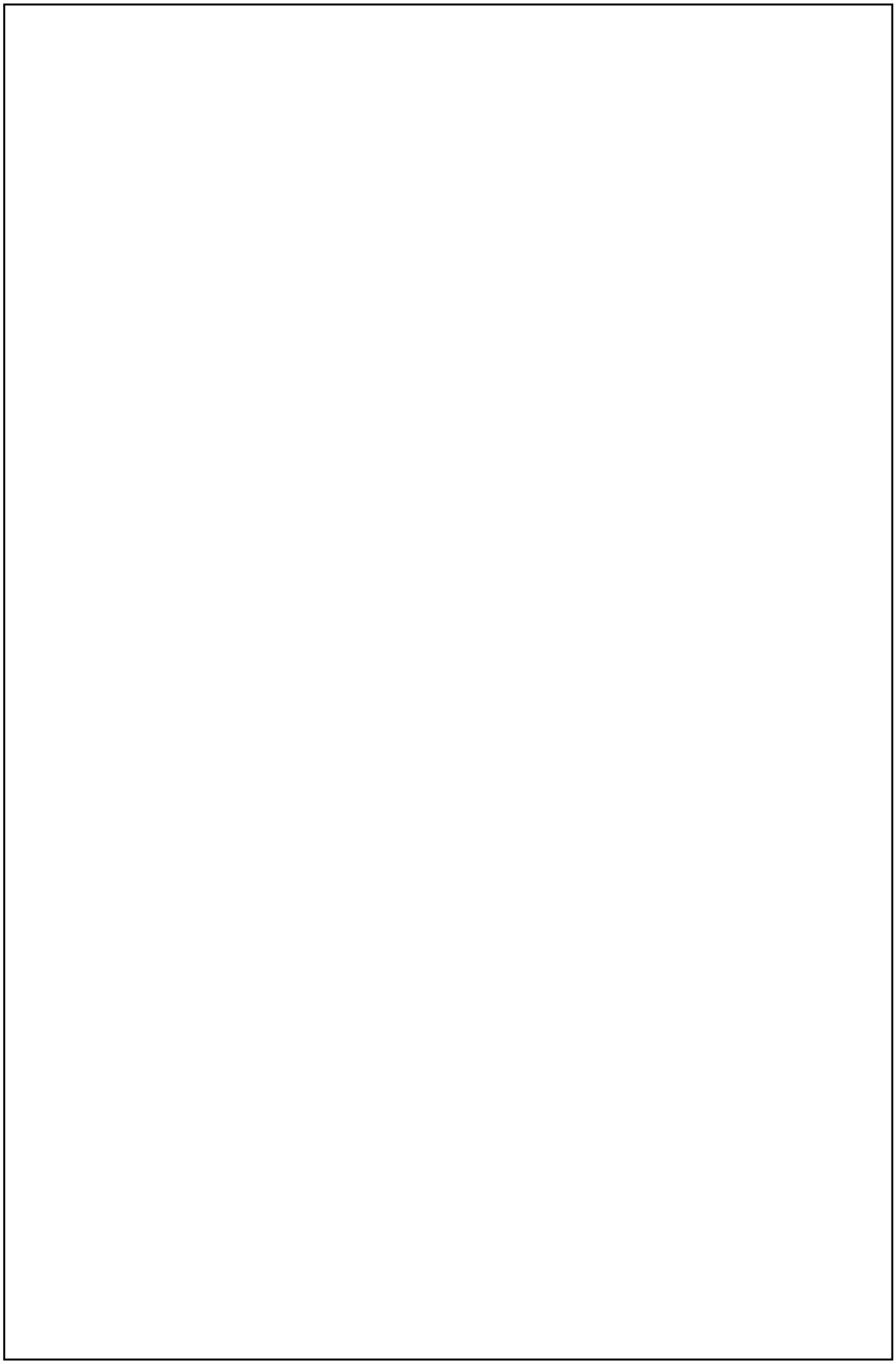


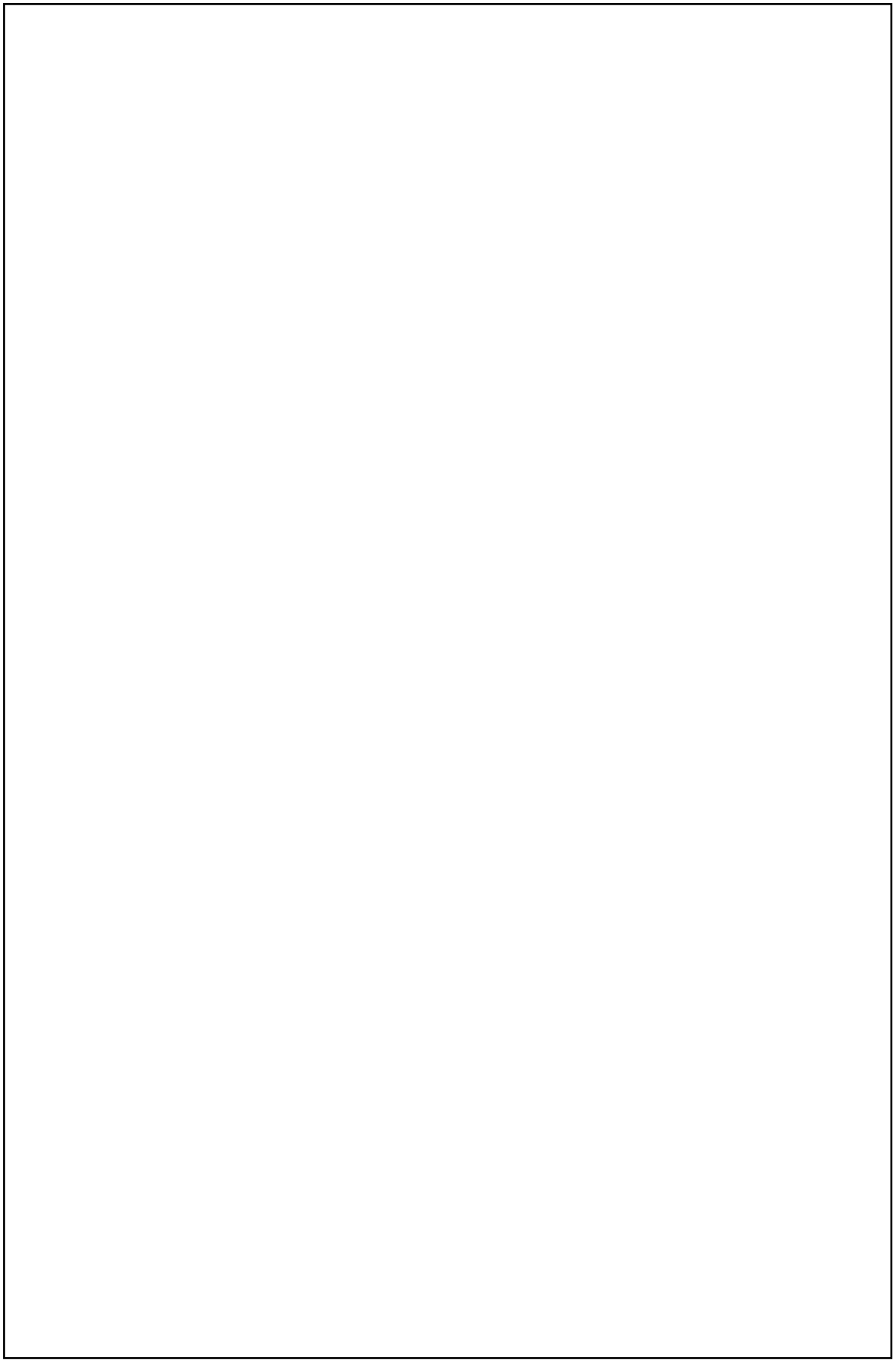


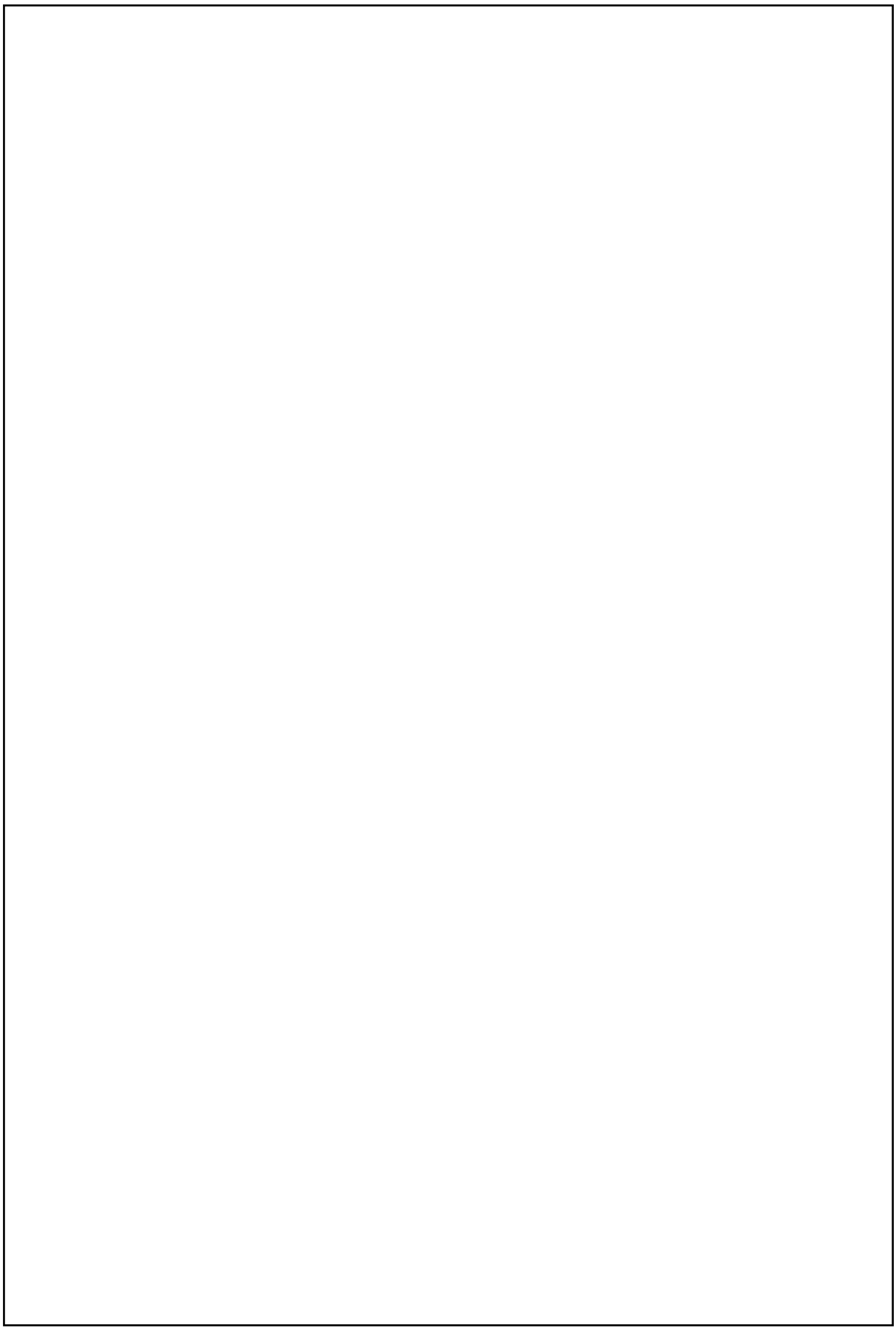


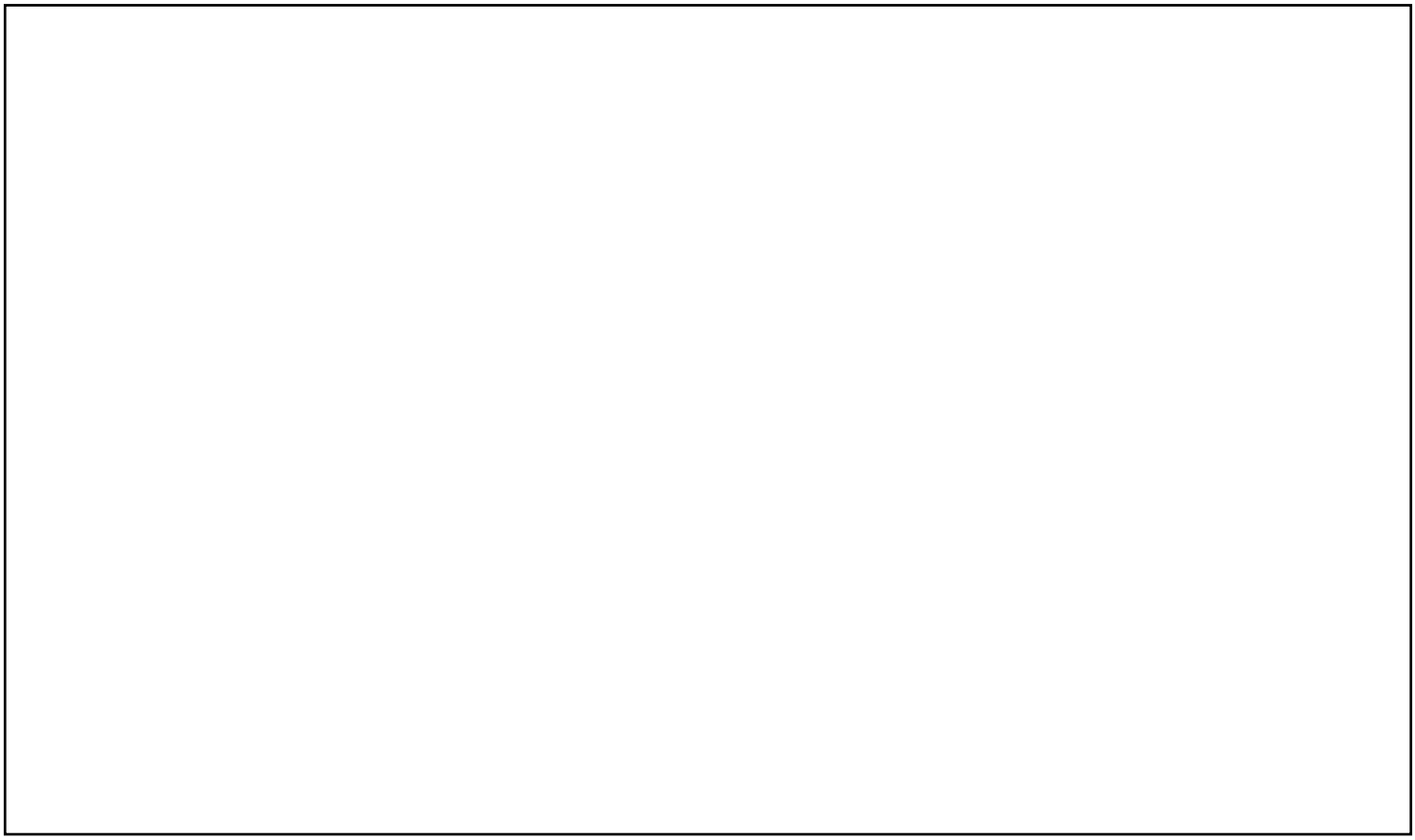


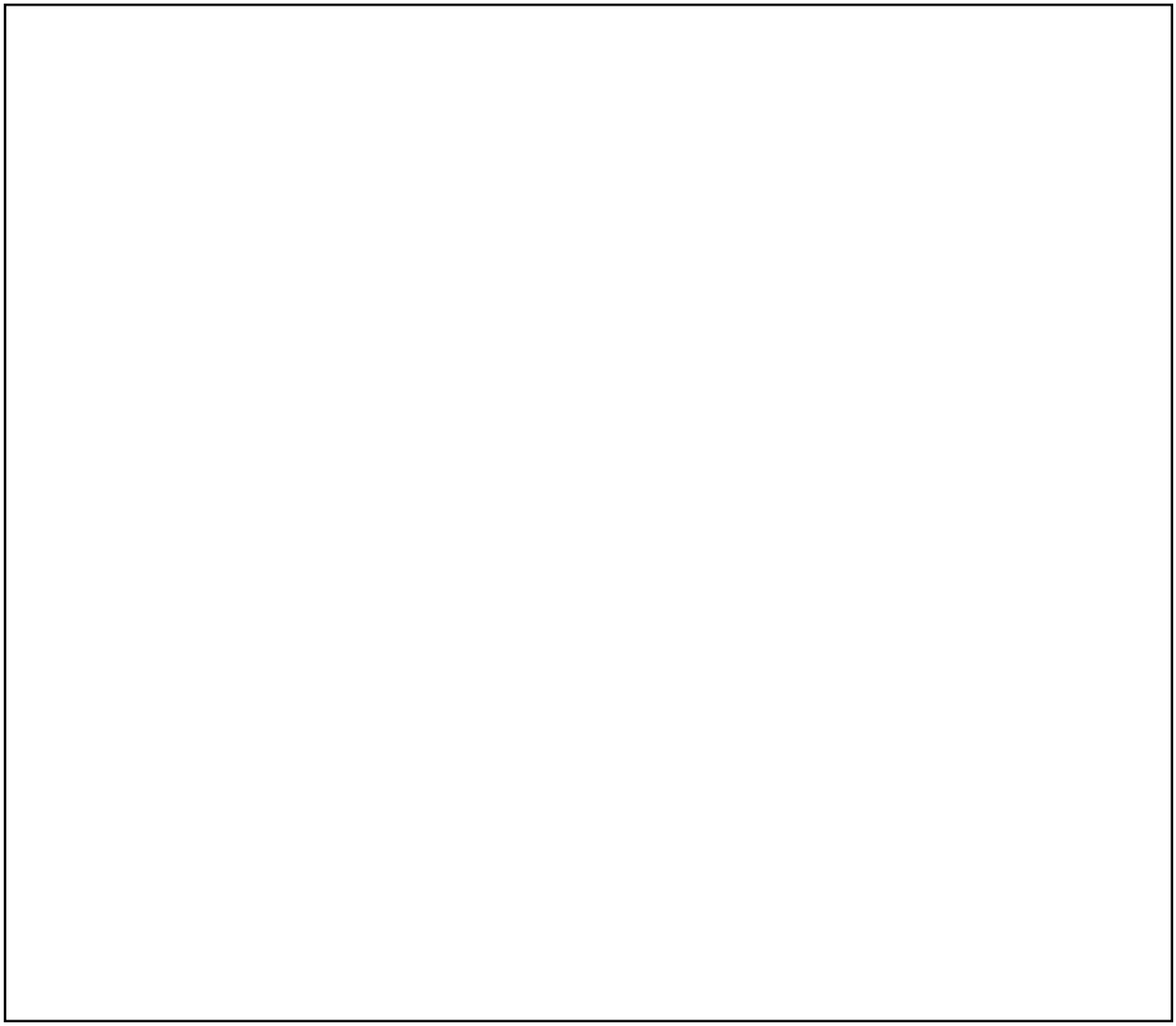


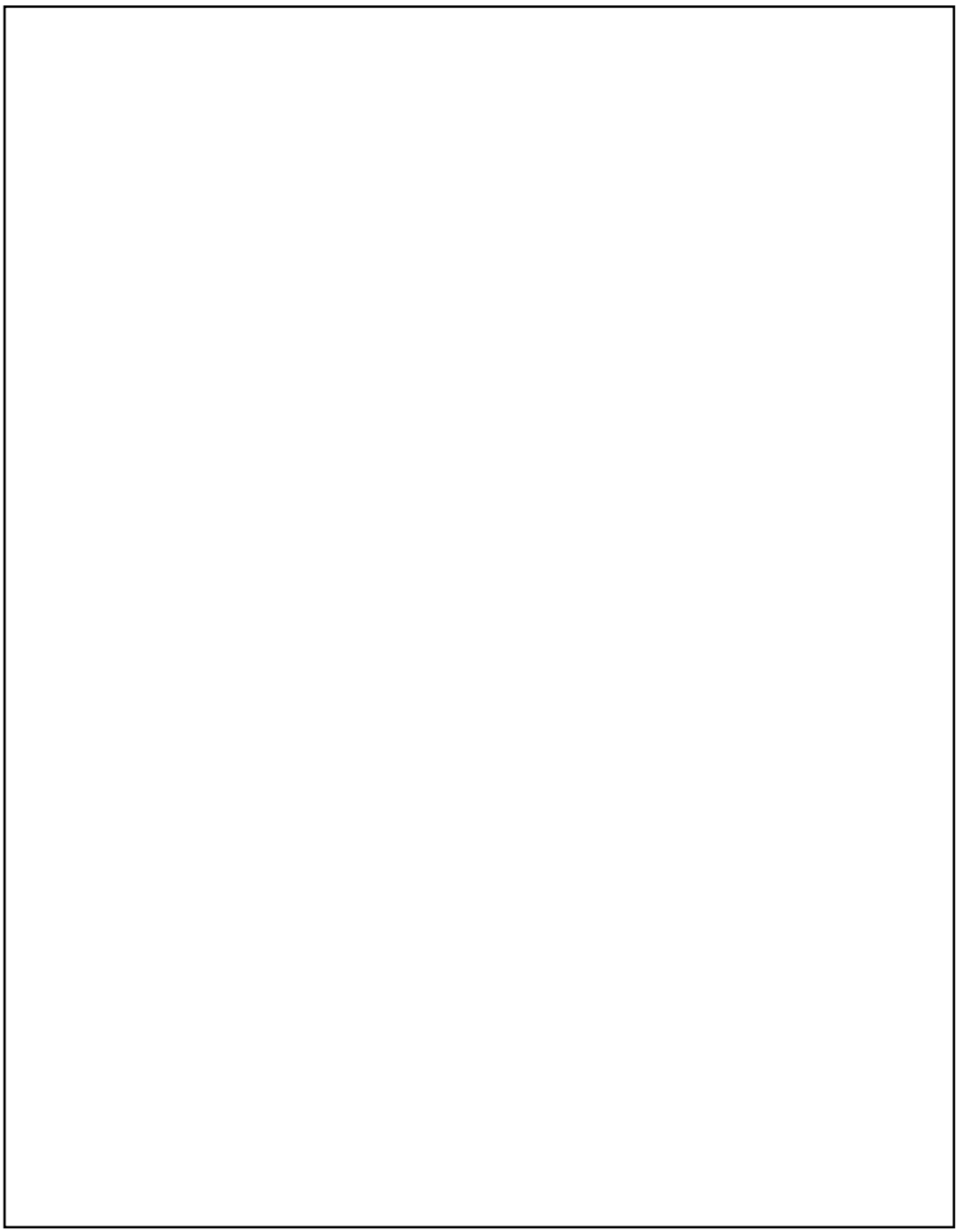


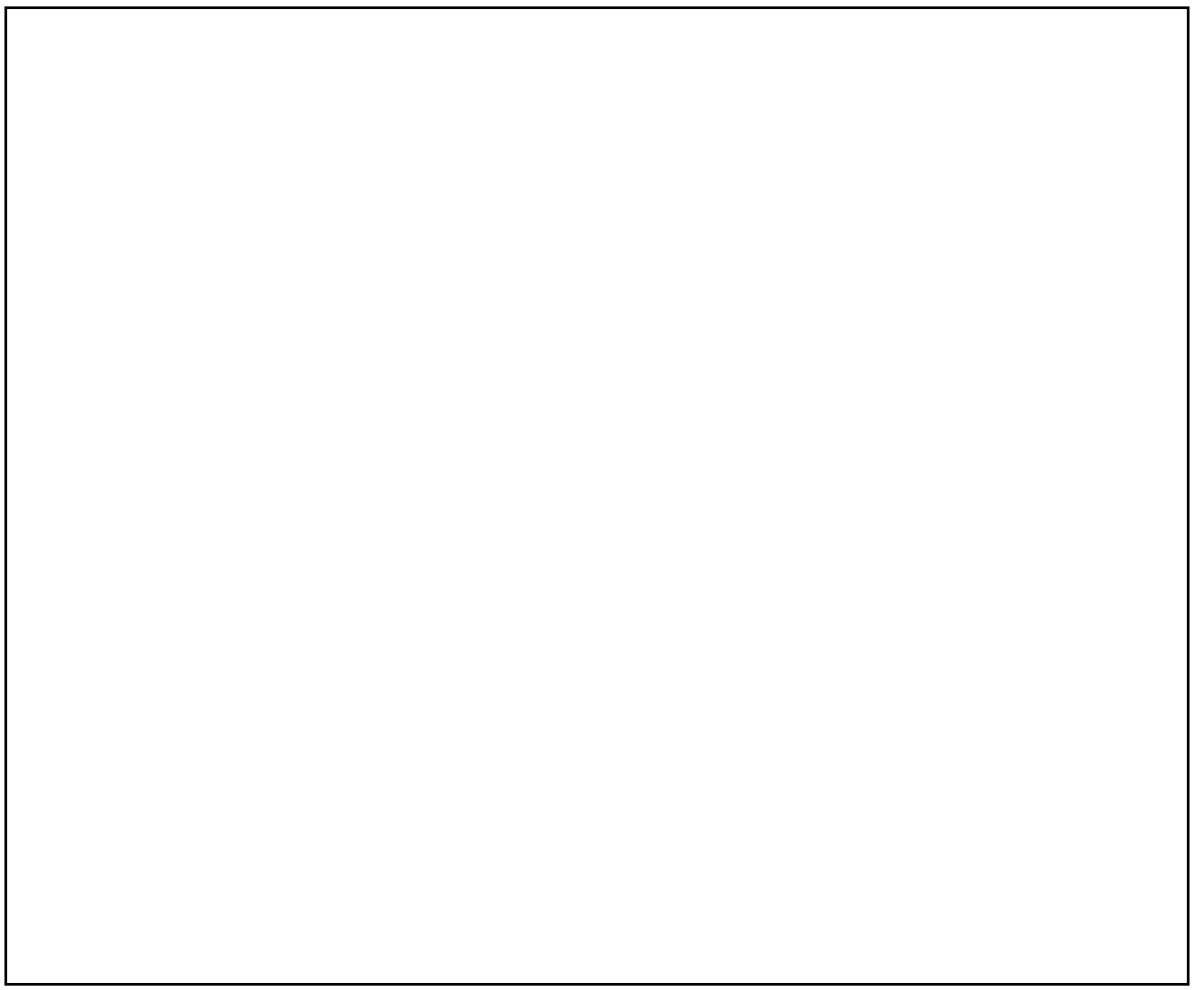


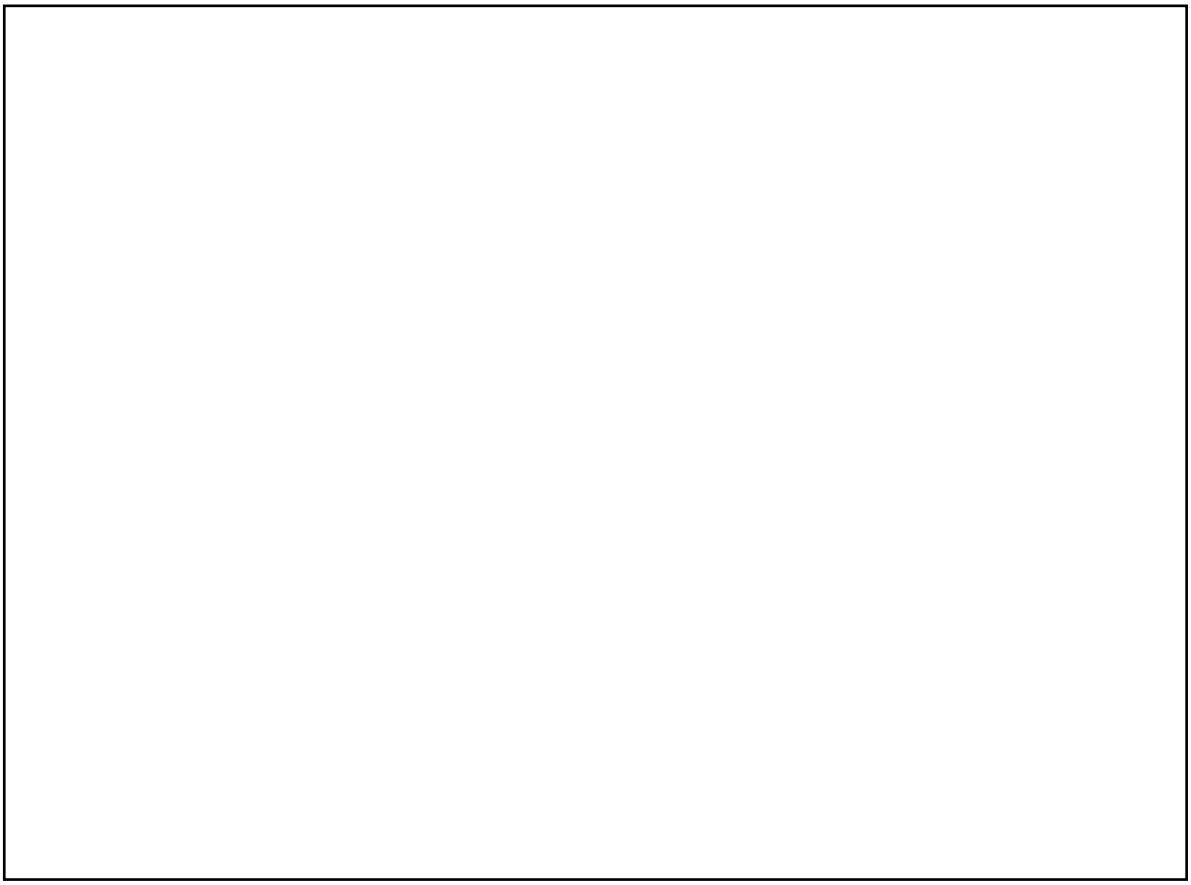


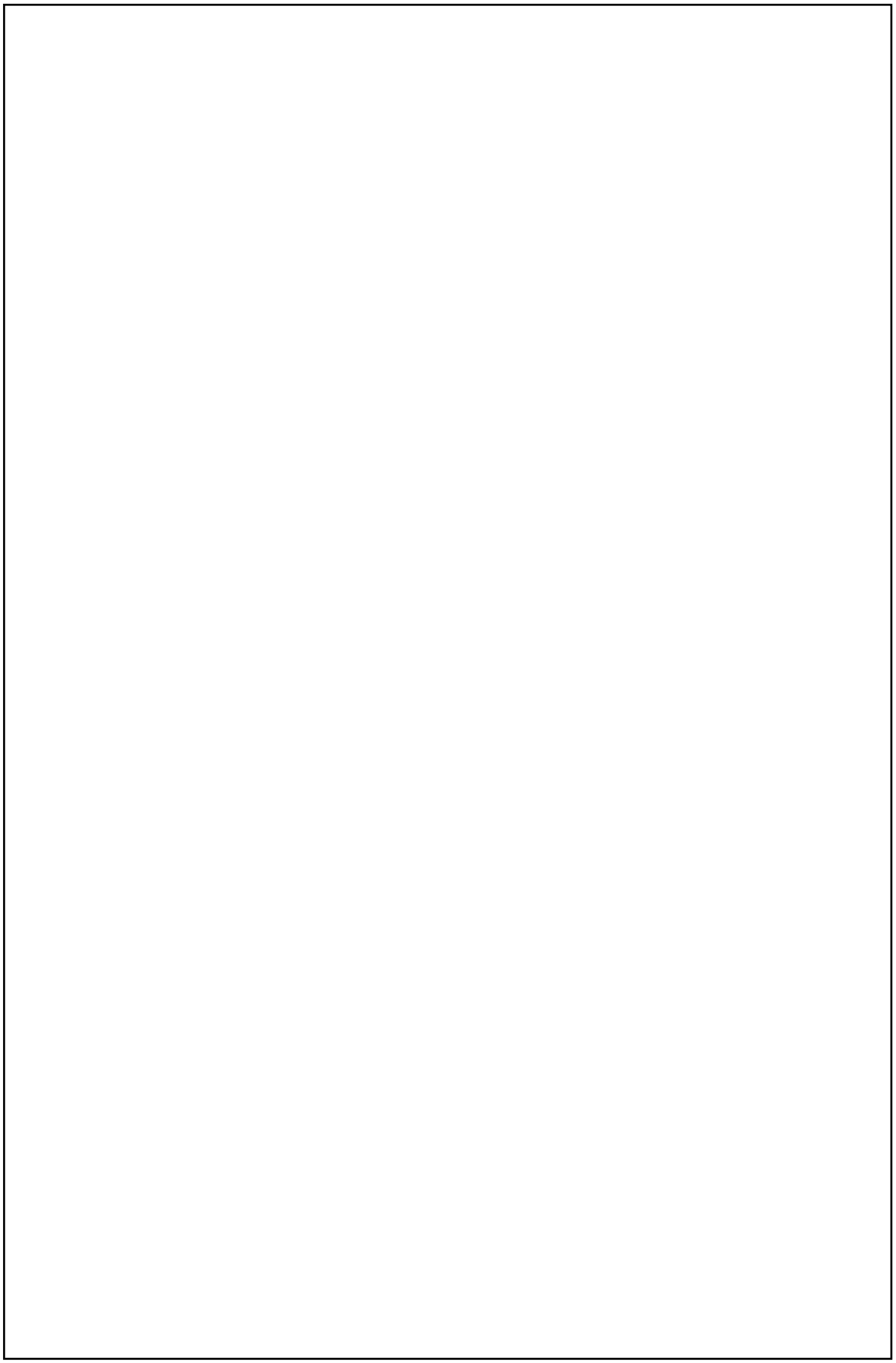


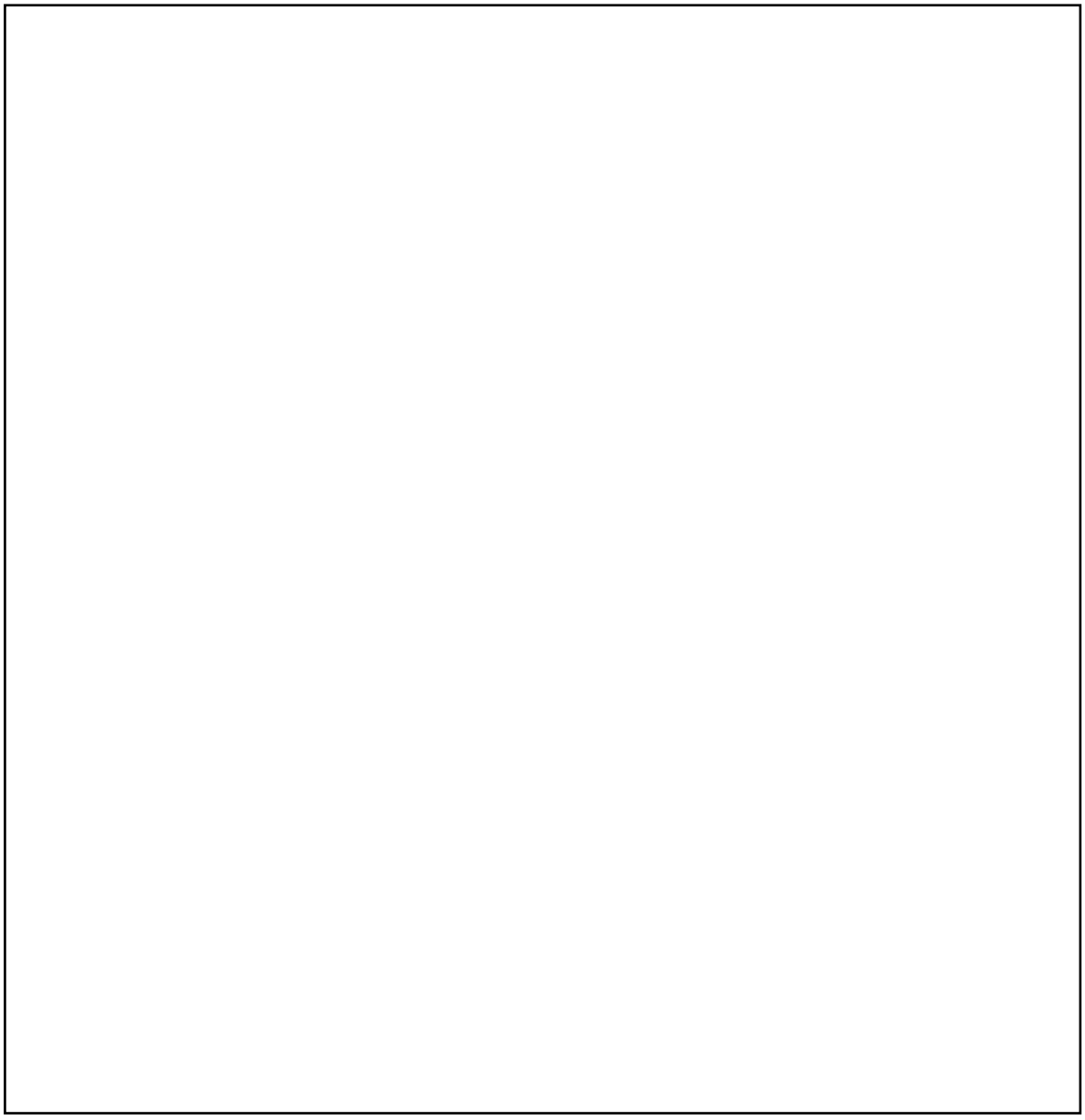


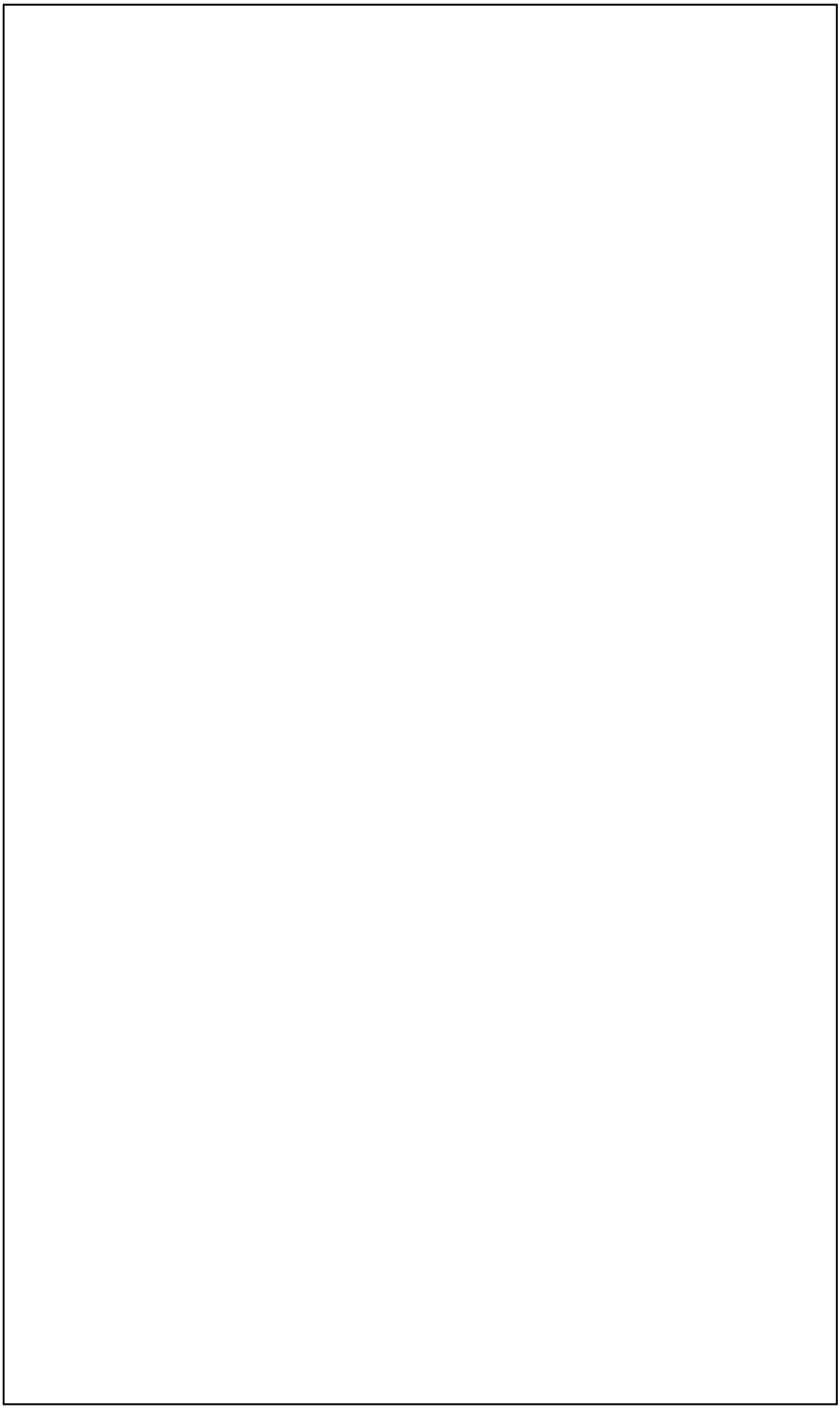


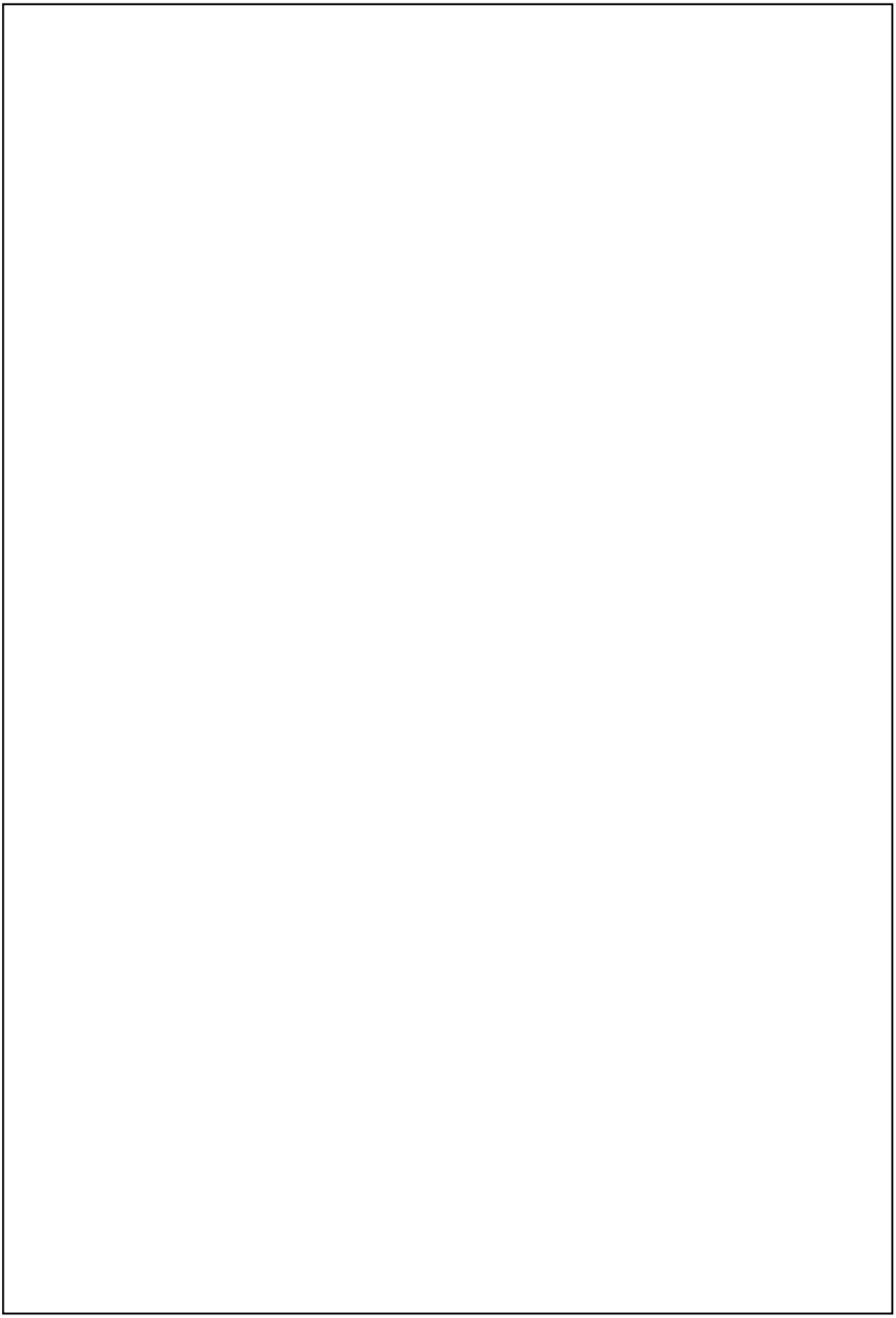


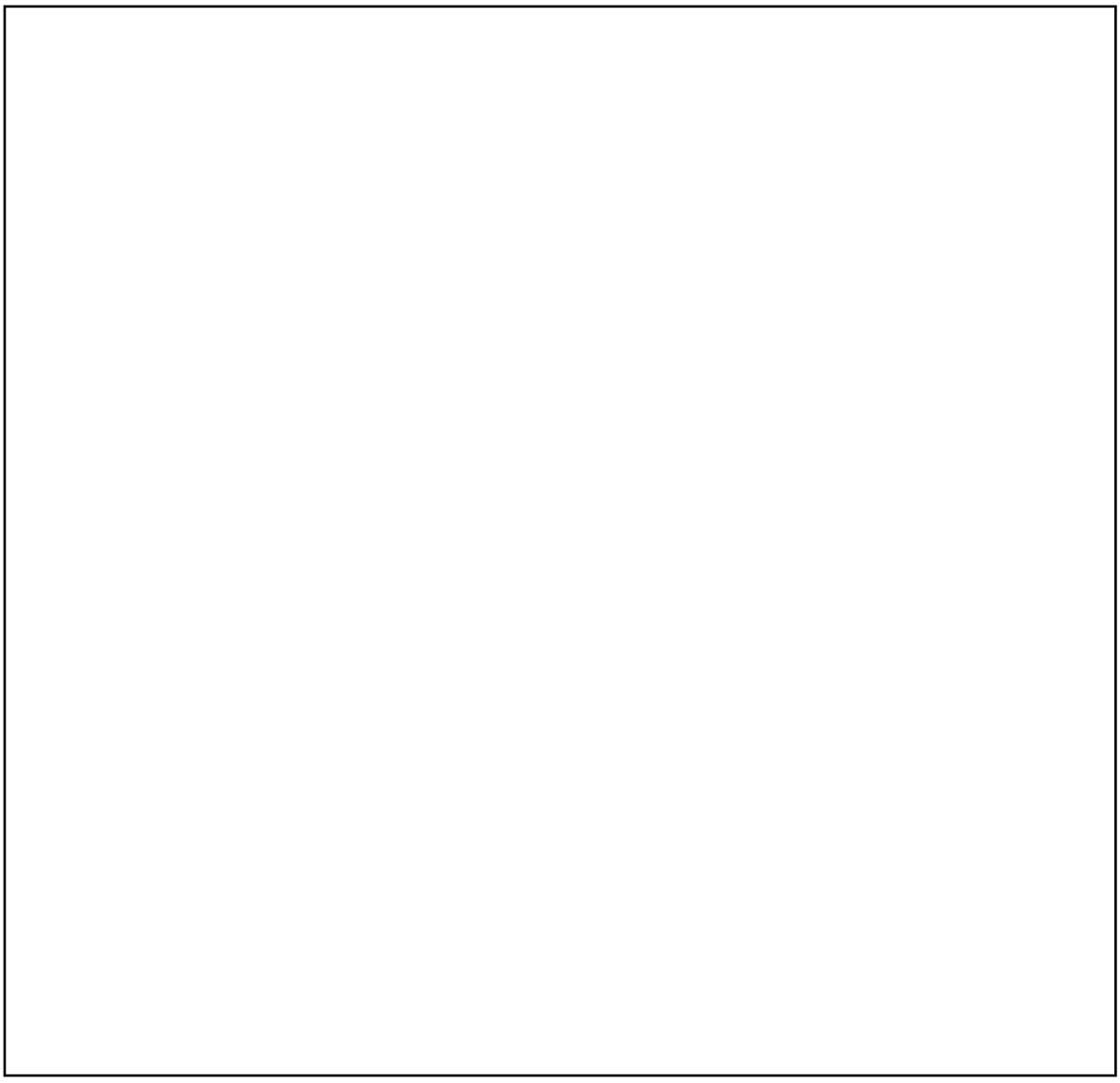


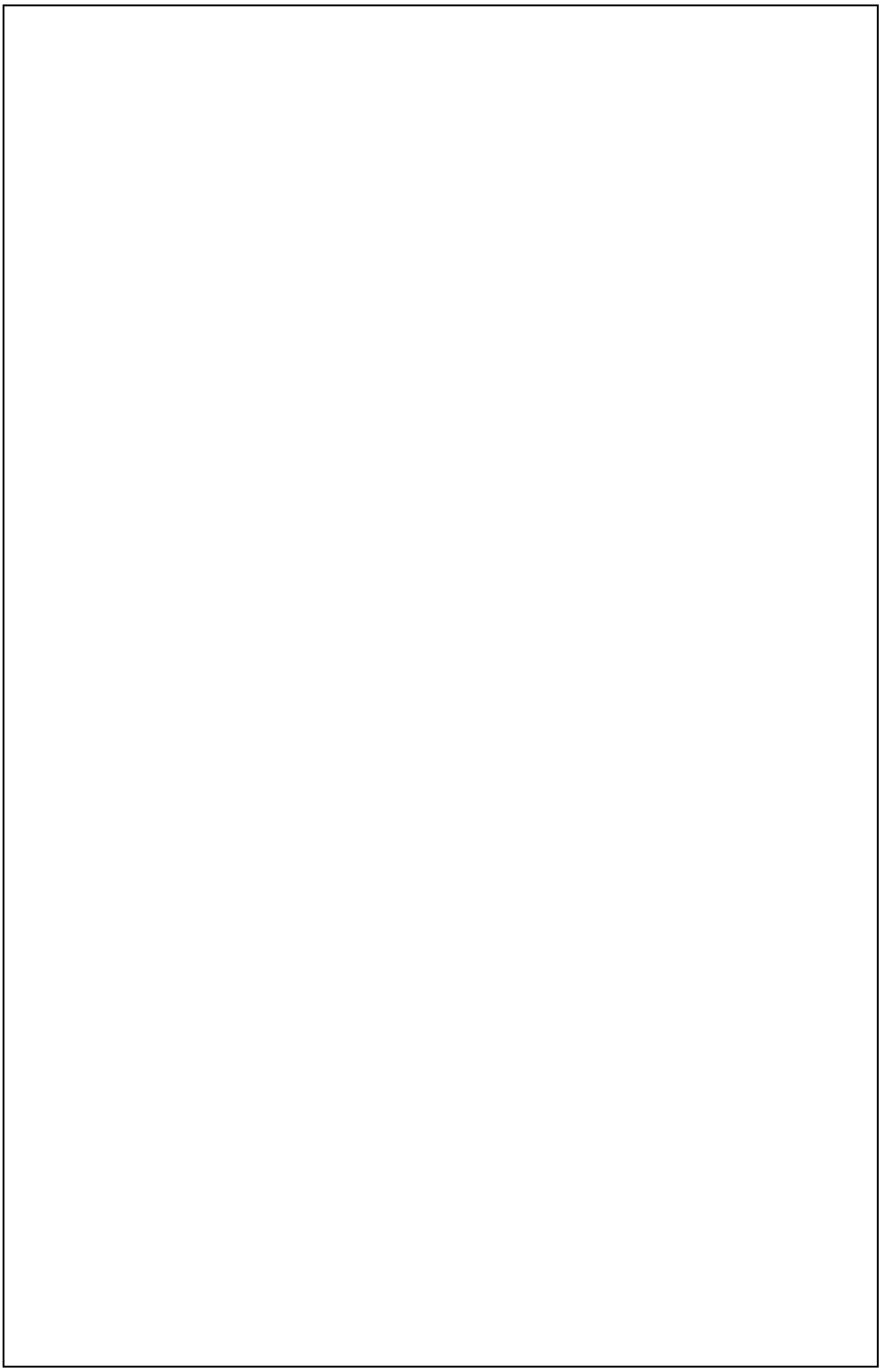












General Discussion

I have shown several genetic factors that are involved in the growth and development of *Arabidopsis thaliana*. In the first chapter, I reported that the *ACL1* gene was identical to *SRFR1*, which negatively regulates defense responses against pathogens. Constitutive activation of defense signaling pathway induced by *SNC1* was found to cause the severe stunted growth such observed in the *acl1-1* mutant. And in the next chapter, HD-ZIP IV genes, whose function in the plant development had been unknown, are suggested to be involved in the proper development and determination of organ identity in flowers. As far as I had investigated, it is uncertain that the phenotype of the HD-ZIP IV mutants is related to the perception of the information from the outer environment like pathogen infection. However, in the analysis of the *acl1* mutants, it became clear that the *ACL1* gene is a key regulator of plant growth correlated with the resistance pathway against pathogens, whose activity is also influenced by temperature and nutrient condition in the growth media.

At present, I have only identified the genetic factors or mutations as an input, and plant growth phenotype as an output of the signaling pathways that regulate plant growth and development. I expect further studies would reveal the molecular mechanisms that link these inputs and outputs, and more details in the co-operative regulation of plant growth and development by genetic and environmental factors. In molecular aspects, lipid metabolism might be related to both of the signaling pathways involved in the growth of the *acl1* and HD-ZIP IV mutants. *EDS1* and *PAD4*, which are

essential for defense response pathways via TIR-NBS-LRR R genes, encode acetyl hydrolase with homology to eukaryotic lipases (Falk *et al*., 1999; Jirage *et al*., 1999). It was therefore suggested that they might play a role in lipid based signaling by hydrolysing a lipid substrate. On the other hand, epidermis-expressed HD-ZIP IV proteins regulate expression of the genes related to lipid metabolism (Abe *et al*., 2003; Wu *et al*., 2011), and it is also possible that extracellular lipid composition is important for the floral development. Although further investigations on the relationships between lipid metabolism and the regulation of defense signaling, or floral development are needed, it would be interesting to examine the involvement of the lipid metabolisms in the regulatory pathways of plant growth and development in future studies.

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